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DIRECTORATE OF DISTANCE EDUCATION

M.Sc. [Microbiology]

II-SEMESTER

364 44

Lab-IV

INDUSTRIAL MICROBIOLOGY & MICROBIAL BIOTECHNOLOGY

SYLLABI-BOOK MAPPING TABLE

Lab-IV – Industrial Microbiology & Microbial Biotechnology

CONTENTS

Industrial Microbiology

Microbial Biotechnology

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UNIT -1 INOCULUM PREPARATION FOR FERMENTATION

Structure

1.1 INTRODUCTION

Inoculum is a microbial preparation, a small active culture used for mass production and also for large scale fermentation of microbial products. Inoculum is otherwise called a small amount of substance containing bacteria from a pure culture which is used to start a new culture or to infect an experimental animal. Inoculum preparation is the part of the fermentation. The development of inoculums ensures the viability of the microorganism and used for any microbe based experimental and/or production purposes by providing a viable biomass capable of high productivity.

An inoculum is considered a whole cell (bacteria), or any part of the microorganism i.e., fungus, such as the spore or mycelium. Inoculum size is the required concentration of expected microorganism for a standard test. It is believed that standard suspension of inoculums consist of required volume with adequate concentration of microbial cells essential for successful operation of either pilot or industrial scale process. Inoculum preparation involves obtaining the organisms in an optimal state that is compatible with inoculation into cell culture, tissue culture, media, and fermentors. Inoculum of the particular microorganism in the specific products such as antimicrobials, enzymes, beverages, drugs, toxins, vitamins, amino acids, organic

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acids, solvents, food products, and recombinant proteins are varied in specific operation.

The prime objective is to achieve a highly active viable biomass in a suitable physiological state for the use of an inoculum. A correct inoculum must be at active sizable growth, free from contamination, and have product-fermenting ability. To prepare suitable good quality inoculums a mother culture and production medium are essential. Inoculum quality is further enhanced by strain improvement and cell immobilization technology.

Further, ideal inoculums is used for, antibiotic susceptibility testing, as described by Clinical Laboratory Standards Institute (CLSI), involve the preparation of pure culture of a single type of organism at specific cell density equivalent to McFarland standard, which constitutes the inoculum. For the industrial fermentation, inoculum must be at desirable cell concentration in required volume is important. Usually 1% to 10% inoculums will be used in industries to reduce the time for product fermentation.

1.2 OBJECTIVES

After going through this Experiment you will be able to;

- Understand the concept of inoculum
- Differentiate vegetative and spore type inoculums.
- Understand the applications of haemocytometer and standard plate count.
- Value the role of inoculums in industrial fermentation.

1.3 ABOUT THIS EXPERIMENT

In this experiment, bacteria *Bacillus* sp. (viable cells) and fungi *Aspergillus niger* (spores) inoculums will be prepare and the concentration of the inoculums will be calculated by the standard procedures as follows.

Haemocytometry

To enumerate the number of cells (or) spores in the suspension, haemocytometric / cell count method is used. The haemocytometer is a counting-chamber originally designed and usually used for counting blood cells. The haemocytometer was devised by Louis-Charles Malassez and consists of a thick glass microscope slide with a rectangular indentation that creates a chamber. This chamber is

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engraved with a laser-etched grid of perpendicular lines. The device is carefully crafted so that the area bounded by the lines is known, and the depth of the chamber is also known. By observing a defined area of the grid, it is therefore possible to count the number of cells or particles in a specific volume of fluid, and thereby calculate the overall concentration of cells in the fluid. A well used type of haemocytometer is the *Neubauer* counting chamber. The counting area of the haemocytometer consists of nine 1 x 1 mm (1 mm^2) squares. These are subdivided in three directions; 0.25 x 0.25 mm (0.0625 mm^2), 0.25 x 0.20 mm (0.05 mm^2) and 0.20 x 0.20 mm (0.04 mm^2). The central square is further subdivided into 0.05 x 0.05 mm (0.0025 mm^2) squares (Fig. 1.1). The raised edges of the haemocytometer hold the coverslip 0.1 mm off the marked grid, giving each square a defined volume.

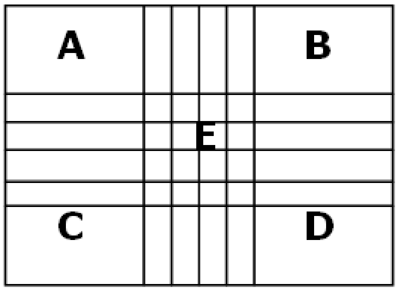


Figure -1.1: Haemocytometer column.

The widely used methods for quantitative determination of bacteria are the standard viable, plate count method. In this standard plate count method, a total viable bacterial population can be determined. The standard plate count method consists of diluting a sample with sterile saline or phosphate buffer until the bacteria are dilute enough to count accurately. That is, the final plates in the series should have between 30 and 300 colonies. Fewer than 30 colonies are not acceptable for statistical reasons (too few to count, TFTC), and more than 300 colonies (too numerous to count, TNTC) on a plate are likely to produce bacterial lawn which cannot be distinguished as distinct colony-forming units **(**CFUs**)**. The assumption is that each viable bacterial can develop into individual single discrete colony (CFU). Thus, the number of colonies should give the number of bacteria that can grow under the incubation conditions employed. A wide series of dilutions (e.g., 10^{-4} to 10^{-10}) is normally plated because the exact number of bacteria is usually unknown. Greater accuracy is

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achieved by plating duplicates or triplicates of each dilution, although we will not be doing that in this exercise (Fig.1.2).

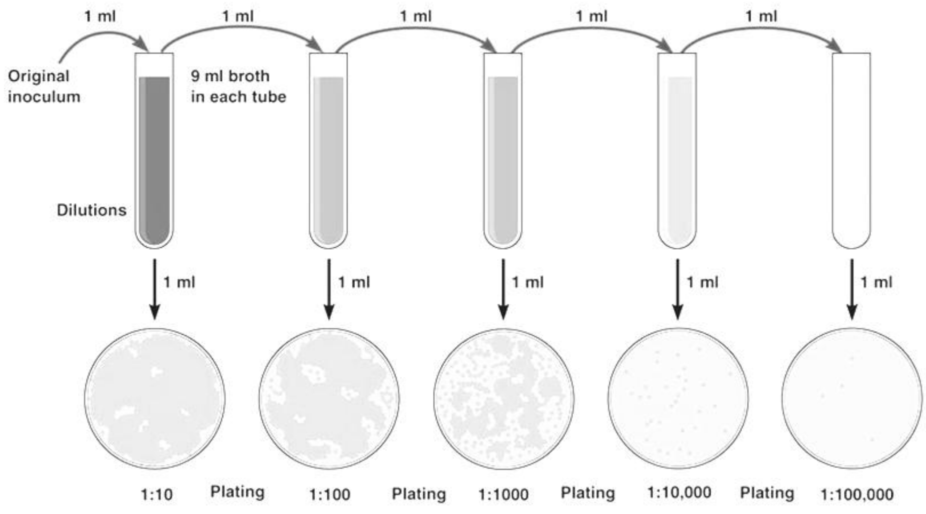


Figure -1.2: Standard Plate Count Method.

1.4 MATERIALS REQUIRED

1. Media: Nutrient agar, nutrient broth, Potato Dextrose Agar (PDA).
2. Sterile saline, Erlenmeyer flask, Pipettes, Spectrophotometer, microscope, haemocytometer, petriplate, etc.
3. Microorganism: *Bacillus* sp., *Aspergillus niger*.

1.5 PROCEDURE

1.5.1 Vegetative inoculum of *Bacillus* sp.

1. To prepare Nutrient agar plates.
2. Inoculate a pure culture of *Bacillus* sp. on Nutrient agar plates by quadrant streaking.
3. Incubate the culture plates at 37^0C for 24 hours.
4. Prepare variable volume of nutrient broth [10 ml in 25 ml flask, 25 ml in 100 ml flask, and 100 ml in 250 ml flask), sterilize by autoclaving at 120^oC for 20 minutes.
5. A single pure colony of the *Bacillus* sp from the Nutrient agar plate will be inoculated in nutrient broth respectively.
6. Incubate the culture flasks at 37^0C for shaken culture method for 24 hours.
7. Take OD value at 595nm against sterile nutrient broth blank.

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8. Measure the concentration of viable cell population in the respective volume of the culture flask – standard plate count method (Fig -1.2).

1.5.1.1 Standard Plate count Method (Fig.1.2)

1. Take a series of 9.0 ml normal saline tubes (8-9 numbers), and label it as 10^{-1}, 10^{-2}, …. 10^{-9}.
2. Add one ml of the Bacillus culture in to the first tube (10^{-1}), mix thoroughly, take one ml from this tube to second tube (10^{-2}) and mix properly.
3. Repeat this process up to the final tube (10^{-9}), and discard 1.0 ml from the final tube.
4. 0.1ml of the diluted bacterial suspension is spread using L-rod on the surface of the respective Nutrient agar plates.
5. Incubate the plates at 37°C for 24 hours and verify the total viable cells as CFU/ml.

1.5.2 Spore inoculums of *Aspergillus niger*

1. Inoculate a pure culture of *Aspergillus niger* on a single spot at the centre of PDA plates.
2. Incubate the culture plates at 32^0C for 48 to 72 hours.
3. After incubation, *Aspergillus niger* colony produces large number of black colour spores.
4. Add 5 ml of sterile distilled water to the plates.
5. Scrap the *Aspergillus* spores; collect the spore suspension in a sterile centrifuge tube.
6. Centrifuge at 10,000 RPM for 10 minutes. Discard the supernatant; resuspend the spore at the required volume of sterile water.
7. Count the number of spores using Haemocytometer method.

1.5.2.1 Measuring Spore Concentration with Hemacytometer

1. Prepare a spore suspension.
2. Prepare a haemacytometer for use.
   a. Carefully clean all surfaces of the haemacytometer and cover-slip.
   b. Take care to ensure that all surfaces are completely dry.
   c. Center the cover slip on the haemacytometer.
3. Pipette approximately 10µl (this volume will vary slightly with the brand of haemacytometer) of the cell suspension into one of the two counting chambers.

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a. Use a clean pipette tip.
b. Be sure that the suspension is thoroughly, but gently, mixed before drawing the samples.
c. Fill the chambers slowly and steadily.
d. Avoid injecting bubbles into the chambers.
e. Do not overfill or underfill the chambers.

4. Count the spores: Count all of the spores in each of the four 0.1 mm^3 corner squares labeled **A** to **D** in Fig 1.1.
5. To determine the number
   1) DO count the spores touching the top or left borders.
   2) DO NOT count the spores touching the bottom or right borders.
6. Determine the Spore Count.
   a. Calculate the total spores counted in the four corner squares.
   b. Calculate the spore count using the equation: spores/ml = (n) x 10^4, where: n = the average cell count per square of the four corner squares counted.

1.6 OBSERVATION & RESULT

1.6.1 Standard Plate Count Method

Dilution	D value at 595 nm (A)	Total CFU/ml (B)
10^{-3}		
10^{-4}		
10^{-5}		
10^{-6}		
10^{-7}		
10^{-8}		

1.6.2 Haemocytometric Count

Dilution	Number of Spores				Total Spores counted (A + B + C + D)
	A	B	C	D	
10^{-3}					
10^{-4}					
10^{-5}					
10^{-6}					
10^{-7}					

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RESULT:

a) The calculated average (n) of cells (CFU)in the given bacterial suspension = __________ CFU/ml.

b) The calculated average (n) of cells (spores) in the given fungal spore suspension = __________ spores/ml .

1.7 LET US SUM UP

In this experiment, you have learnt about the importance of microbial inoculums, objectives, how to prepare the microbial inoculums by haemocytometer and standard plate count procedures and calculation for the amount of viable cells and spores present in the volume of suspension. This experiment might be useful to carryout Industrial Microbiology and Microbial Biotechnology practical.

1.8 UNIT - END EXERCISES

1. State the merits of haemocytometer.
2. Discus the significance of the countable colonies.
3. Explain the principles of standard plate count method.

1.9 SUGGESTED READING

1. D. A. Santos, M. E. S. Barros, J. S. Hamdan. Establishing a Method of Inoculum Preparation for Susceptibility Testing of *Trichophyton rubrum* and *Trichophyton mentagrophytes.* Journal of Clinical Microbiology, 2006, 98–101.
2. Aberkane, A., M. Cuenca-Estrela, A. Gomez-Lopes, E. Petrikkou, E. Mellado, A. Mozo´n, J. L. Rodrı´guez-Tudella, and Eurofung Network. 2002. Comparative evaluation of two different methods of inoculum preparation for antifungal susceptibility testing of filamentous fungi. J. Antimicrob. Chemother. **50:**719–722.

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UNIT -2 SCREENING OF ANTIBIOTIC PRODUCING MICROORGANISMS

Structure

2.1 SCREENING OF ANTIBIOTIC PRODUCING BACTERIA

2.1.1 INTRODUCTION

Antibiotics also known as antimicrobials, a natural substance of biological, synthetic or semi-synthetic origin, used to treat diseases causing by microbes such as bacteria and fungi. The term antibiosis was introduced at the year 1928 by a French microbiologist and later the term “antibiotic” was introduced by Waksman at the year 1942. Based on the targeted microorganisms antibiotics are classified as broad spectrum antibiotics and narrow spectrum antibiotics, and static or cidal by their action. Generally, antibiotics targets on the pathogens are varied; cell wall inhibition, membrane transport inactivation, inhibition of transcription, translation, DNA replication and enzymes participating primary metabolic pathways. For example, antibiotic penicillin inhibits cell-wall (peptidoglycan) biosynthesis by inactivating peptidyl transferase, an enzyme make cross-linking in-between adjacent NAM-NAG of peptidoglycan.

There are huge number of antibiotics screened from the natural sources of microorganisms of the soil and other habitats. A total of more than 5,000 compounds have been screened from soil microbes, specifically more than 55% of known antibiotics are from *Streptomyces* sp. Though they are high, a relatively small group of microorganisms belonging to the genera Penicillium, Streptomyces, Cephalosporium, Bacillus and Micromonospora are important antibiotic producers. The most important antibiotics such as Amino-glycosides, Cephalosporins, Glycopeptides, Macrolides, Penicillin and Tetracyclines are the products of microorganisms. Due to the reality, *Bacillus* sp. has produces several protein antibiotics (peptide), such as bacitracin and polymyxin, which inhibits other Gram positive bacteria more effectively, and it is cheaper than others. Similarly, the antibiotics producing Streptomyces have great interest in antibiotic biology. The steady increase and the occurrence of antibiotic resistance among pathogens during the recent past, hence it is an urgent need to find suitable and more active new antibiotics. Hence, the demand for new antibiotics to cures for life-threatening diseases is growing day by day.

Several antibiotics producing microorganisms are able to grown on the simple artificial media, are used for the exhaustive search for new antibiotic producers. Soil is very complex and diverse environment providing excellent source of antibiotic producing organisms, ranging from 10^5 to 10^9 number of active form of bacteria. In every year, around 500 new antibiotics have been reported, in which >60% of antibiotics are from soil microorganisms.

Bacillus species are Gram positive, aerobic, and rod shaped sporulating bacteria. They are abundantly found in the soil which is capable of producing different biologically active secondary metabolites. Previous studies confirmed that the genus Bacillus produces pharmaceutical and biotechnological important antimicrobial compounds. The species of Bacillus such as *B. brevis* (Tyrothricin, Gramicidin), *B. cereus* (Zwittermicin, Cerexin) *B. circulans* (Circulin), *B. licheniformis* (Bacitracin) *B. laterosporus* (Laterosporin), *B. polymyxa* (Colistin, Polymyxin), *B. pumilus* (Pumulin) and *B. subtilis* (Bacitracin, Polymyxin, Subtilin, Difficidin, Mycobacillin,) are main polypeptide based antibiotics producer. They are active against *Staphylococcus aureus* and *Micrococcus luteus*. Based on the above background information, this experiment is being proposed to isolate and screen antibiotic producing bacteria from the soil sample.

2.1.2 OBJECTIVES

After going through this Experiment you will be able to;

- Understand the concept of antibiosis.
- Know the isolation procedures for antibiotic producing bacteria.
- Understand the applications of antibiotic sensitivity test.
- Know the conformation of antibiotic producers.

2.1.3 ABOUT THIS EXPERIMENT

In this experiment, two different approaches are used for the isolation and primary screening of antibiotic producing bacteria from the soil sample. They are 1) Crowded plate technique and 2) Winkin's method.

1) **Crowded Plate technique:** To screen antibiotic producing organisms, the simplest method is crowded plate technique. In this technique, the sample is diluted in sterile water/ saline (up to 10^{-4}) then spread onto Petri dishes containing agar gel rich in the nutrients the bacteria will need to grow. The dilutions will be crowed with individual colonies on agar surface, i.e. 300 to 400 colonies or more. Colonies producing antimicrobial activity are indicated by clear zone of growth inhibition surrounding the colony. Such colony is later on sub cultured, purified, and afterwards microbial inhibition spectrum is tested against selective microorganisms (Fig.2.1).

2) Wilkins's Method: A Wilking's medium which contains a pH indicating dyes i.e. Bromo-thymol Blue which is green colour at neutral pH but colourless at acidic pH. This method differentiate antibiotic producer from the acid producing bacteria. Those colonies that produce antibiotics give zone of inhibition against sensitive organisms without changing colour surrounding it while in case of acid producing colony the zone of inhibition is due to fall in pH along with colourless area due high acid production which result in lower pH, ultimately change the colour of dye from green to colourless. This technique has some additional advantage that the organism producing antibiotic against the microorganisms of choice can be secondary screened. Here dilutions of sample are applied to agar surface to get well isolated colonies. After the colonies have reached up to few millimetre in size the suspension of test organisms is spread over the surface of agar and incubated. The

antibiotic activity is indicated by zone of inhibition around the antibiotic producing colony.

After the primary screening, antibiosis colonies are transferred to another fresh plate so that they can be further purified and grow. It's entirely possible, of course, that the colony was really just altering the pH of its environment or making some other change that killed other bacteria, rather than secreting an antibiotic, so further tests are needed to confirm that it is indeed an antibiotic-producing strain. Nonetheless, the crowded plate technique was sometimes helpful in identifying microorganisms that could serve as sources of new antibiotics.

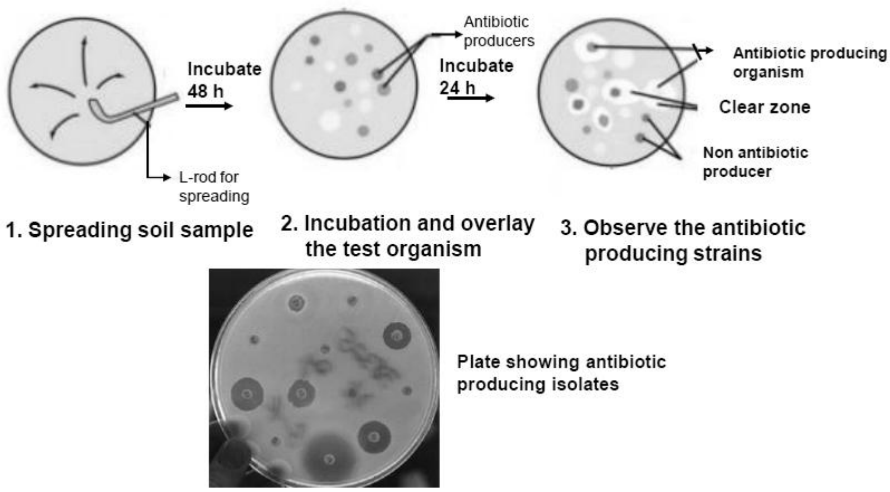


Figure- 2.1: Screening of antibiotic producing bacteria.

Confirmation of antimicrobial activity

There are number of methods used for assay the antibiotic activity against the sensitive microbes. The cross –streak and agar diffusion methods are commonly used for testing the activity of an organism against the test strains or the compounds against the target organisms. The first one method is used for verification of antibiotic producing microbes, and the second is for quantifying the ability of antibiotics to inhibit bacterial growth.

2.1.4 MATERIALS REQUIRED

1. A set of five test tubes (13x10 cm) each tube filled with 5 ml of sterile nutrient broth.
2. Nutrient agar plates.

3. For Wilkins' method, nutrient agar with 0.01% Bromo-thymol Blue as pH indicator (pH 7.0 ±0.1)
4. Sterile water/saline blanks in 13 x 1 cm test tubes, test tubes
5. Muller Hinton (MH) Agar plates / MH broth
6. **Equipments:** Bunsen burner, sterile 1-ml pipettes, L-rod, 10 ml pipettes, mechanical pipetting device, glassware marking pencil, test tube rack.
7. **Test organism**: *E.coli, S.aureus, Pseudomonas aeruginosa* and *Streptococcus epidermidis*
8. **Sample:** Soil sample

2.1.5 PROCEDURE

2.1.5.1 Day -1: Screening of antibiotic producer

1. Three to five sterile test tubes filled with 10 ml of distilled water is taken and labeled the tube.
2. A soil suspension is made by adding 1 g of the soil sample in 10 ml of distilled water (Tube-1) and vortex. Mix well and allow the soil particle to settle down.
3. 1 ml of this solution is taken and transferred to the second test tube and vortex, and from second to third vice versa.
4. Pour 15 ml of sterile nutrient agar media in sterile Petriplate and allow solidifying it.
5. Spread 0.1 ml of inoculum on sterile nutrient agar plate with glass spreader.
6. Incubate the plate at room temperature for 24 to 48 hours.
7. Prepare soft agar (10 ml), sterilize the media and cool at 45^{o}C and add 0.1 ml of test organism individually. Mix the content properly and pour it on the bacteria already grown.
8. Allow to hardening. Incubate the plates at 37^{o}C for 24 hours.
9. Observe the growth inhibition of test organisms (clear zone) surrounding its vicinity, i.e. antibiotic producing colony. Record the results.

(OR)

2.1.5.2 Wilkins's agar plate

1. Spread 0.1 ml of inoculum from each dilution on sterile Wilkins's agar plate.
2. Incubate at 37 ^{o}C for 24 hrs.

3. After 24 hours observe the plate for growth of different organism.
4. Incubate about 2 ml of test culture into Wilkins's broth/ sterile melted Wilkins's top agar previously cooled at 45 °C. Mix it well and pour it over the base Wilkins's agar (bottom agar).
5. Allow it to solidify and incubate at 37 °C for 24 hrs.
6. After 24 hrs observe the plates for colonies showing inhibition of growth of test organism surrounding its vicinity, i.e. antibiotic producing colony.
7. Mark the colony, write colony characteristics, perform Gram's staining, and sub culture it for secondary screening.

2.1.5.3 Day-2: Isolation and pure-culture preparation of antibiotic producers

1. Observe the plate at different intervals and look for the colony which shows the clear inhibition zone surrounding the colony i.e. growth of inhibition.
2. Pick up such colony; inoculate on fresh nutrient agar media by streak plate to prepare pure culture of the isolates.
3. Incubates the culture plates for 24 hours at room temperature.

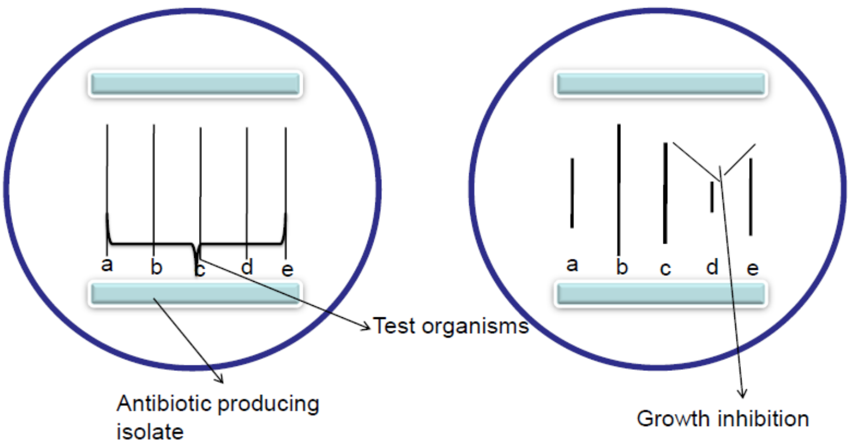


Figure - 2.2: Conformation of antibiotic producing isolates by cross streak method.

2.1.5.4 Day-3: Verification of antibiotic producer by cross-streak method

1) Prepare Muller Hinton agar plates.
2) Inoculate the antibiotic producing isolates by a simple straight line streak at both sides of the plate (as shown in the Fig -2.2). Incubate the plate for 5 days.

3) Streak the sensitive organism in the same plate in between the antibiotic producing isolates in perpendicular, parallel streaks each with different test microbes (as shown in the Fig -2.2).
4) Incubate the culture plates at room temperature for 12 to 24 hrs.
5) Observe the visible growth inhibition at the sensitive organisms. Record the results.
6) Verify the antibiotic production by well diffusion/ agar diffusion method [described in protocol -2, secondary screening of antibiotic producer].

2.6 OBSERVATION & RESULT

2.6.1 Screening of antibiotic producing bacteria

Dilution	Total antibiosis colonies against			
	E.coli	*S.aureus*	*Pseudomonas aeruginosa*	*Streptococcus epidermidis*
10^{-1}				
10^{-2}				
10^{-3}				
10^{-4}				
10^{-5}				

2.6.2 Wilkins's agar plate screening of antibiotic producing bacteria

Dilution	Total antibiosis colonies			
	E.coli	*S.aureus*	*Pseudomonas aeruginosa*	*Streptococcus epidermidis*
10^{-1}				
10^{-2}				
10^{-3}				
10^{-4}				
10^{-5}				

2.6.3 Result

a) The number of antibiotic producing bacteria against *E.coli* = ___________ CFU/ml.
b) The number of antibiotic producing bacteria against *S.aureus* = ___________ CFU/ml.
c) The number of antibiotic producing bacteria against *P.aeruginosa* = ___________ CFU/ml.

d) The number of antibiotic producing bacteria against *St.epidermidis* = __________ CFU/ml.

2.2 SECONDARY SCREENING OF ANTIBIOTIC PRODUCING MICROORGANISMS

2.2.1 INTRODUCTION

Primary screening helps in the detection and isolation of microorganisms from the natural substrates that can be used for industrial fermentations for the production of compounds of human utility, but it cannot give the details of production potential or yield of the organism. Such details can be ascertained by further experimentation. This is known as secondary screening, which can provide broad range of information related to the**:** 1) Ability or potentiality of the organism to produce metabolite that can be used as an industrial organism, 2) The quality of the yield product, 3) The type of fermentation process that is able to perform, 4) Elimination of the organisms, which are not industrially important.

To evaluate the true potential of the isolated microorganisms both qualitative and quantitative analysis are generally conducted. The sensitivity of the test organism towards a newly discovered antibiotic is generally analysed during qualitative analysis, while the quantum yield of newly discovered antibiotic is estimated by the quantitative analysis.

Microorganisms isolated in the primary screening are critically evaluated in the secondary screening so that industrially important and viable potentialities can be detected as; 1) new or novel compound identification, 2) Primary requirements of the isolates (nutrients & other growth factors), 3) detect genetic stability, 4) stability of the synthesized product, 5) productivity, 6) physical and chemical properties of the product, 7) any toxic metabolite produced by the isolate, 8) fermentation economics, 9) complete identification of the isolate with its commercial value, etc.

Several secondary screening methods are available which gives very useful information relating to the newly isolated microorganisms that can be employed in fermentation processes of commercial value. These screening tests are conducted by using petri dish containing solid media or by using flasks or small fermenters containing aqueous media. Each method has some advantages and disadvantages. Sometimes both the methods are employed simultaneously. However, liquid culture

media method is more sensitive than agar plate method because it provides more useful information about the nutritional, physical and production responses of an organism to actual fermentation production conditions.

Liquid Medium Method: This method is generally employed for further screening to determine the exact amount of antibiotic produced by a microorganism. Erlenmeyer flasks containing highly nutritive medium are inoculated with productive strains and incubated at room temperature for 3 to 7 days to produce the antibiotic in an optimum quantity. Collect the cell-free supernatant and test the antibiotic productivity through the agar well diffusion and broth micro dilution methods by using its sensitive test organisms.

2.2.2 Objectives

- To evaluate the potentials of antibiotic producing bacteria against the pathogens.

2.2.3 ABOUT THIS EXPERIMENT

Principle: Agar diffusion method

In this experiment, the pure culture of the primary isolate is inoculated in liquid culture medium and incubated for 3 days. After the incubation the culture supernatant should be collected and test the quantity of antimicrobial substance produced by the organism. For this, the agar diffusion technique is commonly used for determination of minimum inhibitory concentration (MIC) in solid media. It involves the application of antibiotic solutions at different concentrations to cups, wells or paper discs, placed on the surface of or punched into agar plates seeded with the test bacterial strain. Antibiotic diffusion from these sources into the agarose medium leads to inhibition of bacterial growth causes the formation of clear 'zones' without bacterial lawn. The diameter of these zones increases with antibiotic concentration.

2.2.4 MATERIALS REQUIRED

1. Microorganism: Antibiotic producing *Bacillus* sp. (mother culture); sensitive strains: *Proteus mirabilis, E.coli, Pseudomonas sp.* and *Staphyllococcus aureus.*
2. Media: Muller Hinton agar, Nutrient broth

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3. Others: Conical flask, shaker, inoculation loop, Laminar flow hood, Bunsen burner, test tubes, centrifuge, sterile bacteriological filter, Standard filter paper disc.

2.2.5 PROCEDURE

a) Antibiotic Production

1. Prepare 100 ml of nutrient broth / fermentation media in 250 ml Erlenmeyer flask and sterilize by autoclaving.
2. Cool the media at room temperature.
3. Inoculate the antibiotic production medium (1%, v/v) with the pure culture of the log phase primary culture (~10^6 cfu/ml).
4. Incubate at room temperature for 12 hours. After that, incubate the culture flask at rotary shaker for 3 days, at 125 rpm.

b) Preparation of cell free supernatant

1. After the antibiotic fermentation, transfer 25 ml of the fermented media in to a sterile 30ml centrifuge tube.
2. Centrifuge the content at 6000 rpm for 20 min.
3. Transfer the clear supernatant to fresh sterile tube.
4. Further, pass the supernatant through sterile bacteriological filter and collect the antibiotic filtrate aseptically.

c) Agar diffusion method

1. Take sterile standard filter paper disc (6mm dia), impregnate with a known volume of antibiotic culture filtrate (0, 10, 20, 30, 40, …100µl/disc). Aspirate the disc at room temperature aseptically.

2. Prepare muller Hinton Agar plates.

3. Using sterile swap inoculate (spread) 18-24 h log phase test culture strains* on the MHA plates.

4. Place the antibiotic preloaded disc on a Mueller-Hinton (MH) agar plate. Wait for few minutes to wet the disc. Label the plates and the disc information's at the bottom of the MHA plates.

5. Incubate the culture plates at 37oC for 24 hours.

6. Observe the clear zone around the disc and measure with a caliper or ruler.

*Sensitive strains should be maintained as 0.5 McFarland standards.

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BOX-2.1: McFarland standards

McFarland standards are suspensions of either barium sulfate or latex particles that allow visual comparison of bacterial density (Fig. 2.3). Commercially prepared standards are available for purchase from companies such as Remel or BD BBL. These often include a Wickerham card, which is a small card containing parallel black lines. A 0.5 McFarland standard is equivalent to a bacterial suspension containing between 1 x 10^8 and 2 x 10^8 CFU/ml of *E. coli*.

A 0.5 McFarland standard may be prepared in-house as describe below.

1. Add a 0.5-ml aliquot of a 0.048 mol/liter $BaCl_2$ (1.175% wt/vol $BaCl_2 \cdot 2H_20$) to 99.5 ml of 0.18 mol/liter H_2SO_4 (1% vol/vol) with constant stirring to maintain a suspension.
2. Verify the correct density of the turbidity standard by measuring absorbance using a spectrophotometer with a 1-cm light path and matched cuvette. The absorbance at 625 nm should be 0.08 to 0.13 for the 0.5 McFarland standard.
3. Transfer the barium sulfate suspension in 4- to 6-ml aliquots into screw-cap tubes of the same size as those used in standardizing the bacterial inoculums.

Tightly seal the tubes and store in the dark at room temperature.

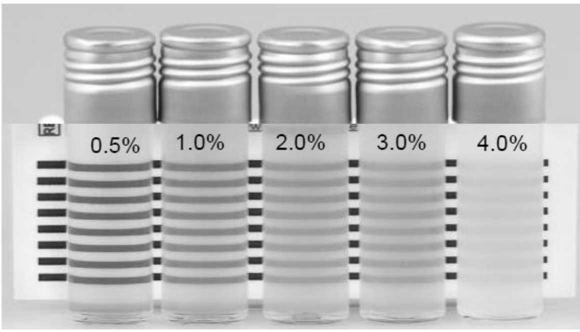


Figure -2.3: McFarland standard.

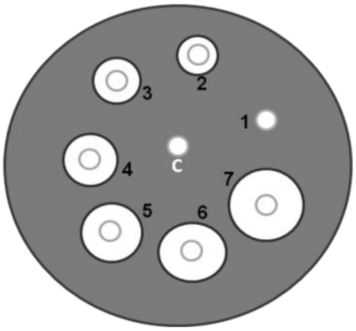


Figure- 2.4: Quantitative determination of antibiotic production by the isolate [1 to 7 different concentrations of antibiotics].

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2.2.6 OBSERVATION & RESULT

Observation: Antibiotic production

Antibiotic Strain No.	*Zone of inhibition (mm) at different volume of the supernatant (µl)*									
	E.coli					*Proteus mirabilis*				
	10 *µl*	20 *µl*	40 *µl*	60 *µl*	80 *µl*	10 *µl*	20 *µl*	40 *µl*	60 *µl*	80 *µl*
1										
2										
3										
4										

Note: Zone size observed in a disk diffusion test has no meaning in and of itself. The interpretation of resistance and susceptibility to antimicrobials is determined through in vivo testing. This information is correlated with zone sizes resulting in the interpretive standards. The current interpretation standards can be found in the Clinical Laboratory Standards Institute Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standards 9th Edition (CLSI standards).

Result

The Isolate _____________, produced __________ amount of antibiotic against_________________.

2.3 LET US SUM UP

In this experiment, you have learnt about the isolation methods of antibiotic producing bacteria by simple crowded plate technique, followed with cross streak analysis. The high antibiotic productivity should be verified using the agar disc diffusion assay procedure. In addition, the method for McFarland standards preparations also studied. This experiment might be very important for searching of new and novel antibiotic producers from the soil and other environmental samples.

2.4 UNIT - END EXERCISES

1. Discuss about antibiosis.
2. How do you isolate non-acid antibiotic producing bacteria? Explain.
3. Explain the CLSI standards protocol for the antibiotic sensitivity test.

2.5 SUGGESTED READING

1. Abebe Bizuye, Feleke Moges, and Berhanu Andualem. Isolation and screening of antibiotic producing actinomycetes from soils in Gondar town, North West Ethiopia. Asian Pac J Trop Dis. 2013 Oct; 3(5): 375–381.
2. Fakhar-un-Nisa Yunus, Zam Zam Khalid, Farzana Rashid, Asfa Ashraf1, Muhammad Naeem Iqbal, Firasat Hussain. Isolation and Screening of Antibiotic producing Bacteria from Soil in Lahore City. PSM Microbiology 2016, 1: 01-04
3. Paul W. Lepp. General Microbiology Laboratory Manual**.** Second Edition. Biol 142, General Microbiology – Spring 2010.
4. Burton E. Pierce and Michael J. Leboffe. Microbiology: Laboratory Theory & Application. Morton Publishing Company; 1 edition (January 1, 2019)
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UNIT -3 SCREENING OF ANTIBIOTIC PRODUCING ACTINOMYCETES

Structure

3.1 SCREENING OF ANTIBIOTIC PRODUCING *Streptomyces* sp.

3.1.1 INTRODUCTION

Secondary metabolites are produced by many microorganisms such as bacteria, actinomycetes, cyanobacteria, and fungi. Among the various groups of microorganisms the actinomycetes occupy a prominent place and have the capacity to produce so many metabolites. Actinomycetes are high G+C containing Gram-positive bacteria but are distinguished from other bacteria by their morphology, physiology and its genomic characters. Actinomycetes are ubiquitous in nature and are widely distributed in natural and man-made environments. They are found in large numbers in soils, fresh waters, marine sediments, mangroves, composts etc. The secondary metabolites of the actinomycetes are diverse depending on the environmental factors and their genetic characters, they have high commercial value. More than 70% of naturally occurring antibiotics have been isolated from different species of *Streptomyces*, is the largest genus known for the production

of many secondary metabolites with different biological activities, such as antibacterial, antifungal, antiparasitic, anticancer and immune-suppressive functions. Streptomyces are antibiotic treasure, less than 2% of the metabolites of the Streptomyces have been identified. To find new and novel antibiotics, scientists are searching new *Streptomyces* sp. from variety of environments. A great number of putative secondary metabolite biosynthesis pathways have been discovered using these genome data which can involve the synthesis of novel metabolites with potential activities. Looking for the new and safe antibiotics to tackle the antibiotic resistance problem, the aim of this experiment is to isolate *Streptomyces* sp. from the humus rich soil and which may produce potent antibiotics against the selective human pathogenic microorganisms.

2.1.2 OBJECTIVES

After going through this Experiment you will be able to;

- Screen the actinomycetes from the soil.
- Screen antibiotic producing actinomycetes like Streptomycetes sp. from soil.
- Know the culture methods of actinomycetes.

2.1.3 ABOUT THIS EXPERIMENT

This experiment has been proposed to isolate and screen antibiotic producing actinobacteria from the soil sample.

The principle of this experiment is similar to the experiment section 2.1. The crowded plate technique with agar overlay can be performed for the screening of antibiotic producing actinobacterial species.

After the primary screening, antibiosis colonies are transferred to another fresh plate so they can be purified and grown in isolation. It's entirely possible, of course, that the colony was really just altering the pH of its environment or making some other change that killed other bacteria, rather than secreting an antibiotic, so further tests are needed to confirm that it is indeed an antibiotic-producing strain. Nonetheless, the crowded plate technique was sometimes helpful in identifying microorganisms that could serve as sources of new antibiotics.

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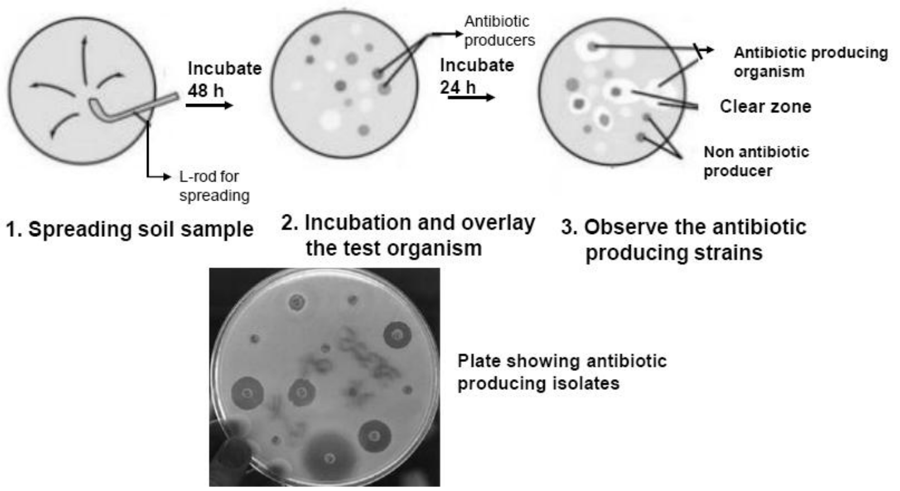


Figure- 3.1: Screening of antibiotic producing Actinobacteria.

2.1.4 MATERIALS REQUIRED

1. Five test tubes (13x10 cm) of each tube filled with 5 ml of sterile nutrient broth.
2. Actinomycete *Isolation* Agar plates / starch casein agar plates10 Nos.
3. Sterile water/saline blanks (10 Nos.) in 13 x 1 cm test tubes, test tubes.
4. **Equipments:** Bunsen burner, sterile 1-ml pipettes, L-rod, 10 ml pipettes, mechanical pipetting device, glassware marking pencil, test tube rack.
5. **Test organism**: *E.coli, S.aureus, Pseudomonas aeruginosa* and *Streptococcus epidermidis.*
6. **Sample:** Soil sample.

3.1.5 PROCEDURE

1. Three to five sterile test tubes filled with 9.0 ml of distilled water is taken and labeled the tube.
2. A soil suspension is made by adding 1 g of the soil sample in 9.0 ml of distilled water (Tube-1) and vortex. Mix well and allow the soil particle to settle down.
3. 1 ml of this solution is taken and transferred to the second test tube and vortex, and from second to third vice versa.
4. Pour 15 ml of sterile Actinomycetes Isolation agar / starch casein agar media in sterile Petri plate and allow solidifying it.

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5. Spread 0.1 ml of inoculum on sterile Actinomycetes Isolation agar plates with glass spreader.
6. Incubate the plate at room temperature for 5 days.
7. Prepare soft agar (10 ml), sterilize the media and cool at 45^{o}C and add 0.1 ml of test organism individually. Mix the content properly and pour it on the bacteria already grown.
8. Allow to hardening. Incubate the plates at 37^{o}C for 24 hours.
9. Observe the growth inhibition of test organisms (clear zone) surrounding its vicinity, i.e. antibiotic producing colony. Record the results.
10. Further, the potent antibiotic producing actinomycetes will be identified by using the methods described in – b given below.

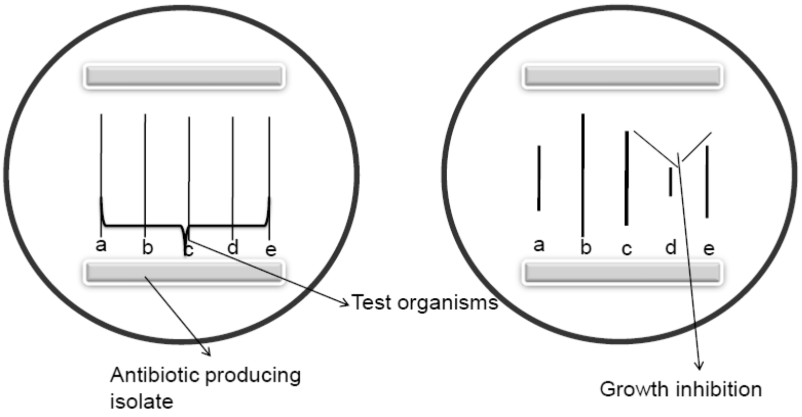


Figure - 3.2: Conformation of antibiotic producing isolates by cross streak method**.**

3.1.5.1 Verification of antibiotic producer by cross-streak method

1) Prepare Actinobacteria isolation agar plates (15ml/plate).
2) Inoculate the antibiotic producing isolates by a simple straight line streak at both sides of the plate (as shown in the Fig -3.2). Incubate the plate for 5 days.
3) Pour Muller Hinton agar (10ml/plate) on the Actinobacteria grown plates by agar overlay method.
4) Streak the sensitive organism in the same plate in between the antibiotic producing isolates in perpendicular, parallel streaks each with different test microbes (as shown in the Fig -3.2).
5) Incubate the culture plates at room temperature for 12 to 24 hrs.
6) Observe the visible growth inhibition at the sensitive organisms. Record the results.

7) Verify the antibiotic production by well diffusion/ agar diffusion method [described in protocol -2, secondary screening of antibiotic producer].

3.1.6 OBSERVATION & RESULT

3.1.6.1 Screening of antibiotic producing actinobacteria

Dilution	Total antibiosis colonies against			
	E.coli	*S.aureus*	*Pseudomonas aeruginosa*	*Streptococcus epidermidis*
10^{-1}				
10^{-2}				
10^{-3}				
10^{-4}				
10^{-5}				

3.1.6.2 Cross streak method

Isolate	Growth Inhibition (+/-)			
	E.coli	*S.aureus*	*Pseudomonas aeruginosa*	*Streptococcus epidermidis*
1				
2				
3				
4				
5				

+ = inhibited; - = no inhibition

3.1.6.3 Result

a) The number of antibiotic producing actinobacteria against *E.coli* = ___________ CFU/ml.

b) The number of antibiotic producing actinobacteria against *S.aureus* = ___________ CFU/ml.

c) The number of antibiotic producing actinobacteria against *P.aeruginosa* = ___________ CFU/ml.

d) The number of antibiotic producing actinobacteria against *St.epidermidis* = ___________ CFU/ml.

3.2 IDENTIFICATION OF ANTIBIOTIC PRODUCING *STREPTOMYCETES* SP.

3.2.1 IDENTIFICATION SYSTEM

Morphological identification of the *Actinobacteria* at the genus and species levels is mainly by microscopic examinations. Mycelial fragmentation can be regarded as a special form of vegetative reproduction (or formal asexual spores). *Actinobacteria* exhibit a wide variety of morphologies, differing mainly with respect to the presence or absence of a substrate mycelium or aerial mycelium, the color of the mycelium, the production of diffusible melanoid pigments, and the structure and appearance of their spores (Fig. 3.3).

(i) Mycelial morphology: Actinomycetes produces aerial hyphae except *Sporichthya* sp., *Actinobacteria* form a substrate mycelium in both submerged and solid-grown cultures. However, on solid surfaces, many differentiate to form aerial hyphae, whose main purpose is to produce reproductive spores. The substrate mycelium develops from outgrowth of a germinating spore. The branching substrate mycelium is often monopodial, but in some rare cases, *Actinobacteria*, such as *Thermoactinomyces*, exhibit dichotomous branching. On the other hand, members of the *Micromonosporaceae* family produce an extensive substrate mycelium with an absent or rudimentary aerial mycelium.

In general, *Actinobacteria* exhibit a wide variety of morphologies, including coccoid (*Micrococcus*) and rod-coccoid (*Arthrobacter*), as well as fragmenting hyphal forms (*Nocardia* spp.) and also forms with permanent and highly differentiated branched mycelia (e.g., *Streptomyces* spp., *Frankia*). Rhodococci form elongated filaments on the substrate and do not produce a true mycelium, while corynebacteria do not produce mycelia at all. Other *Actinobacteria*, the filaments grow at the apex instead of by lateral wall extension. *Actinobacteria* belonging to the genus *Oerskovia* are characterized by the formation of branched substrate hyphae that break up into flagellated motile elements.

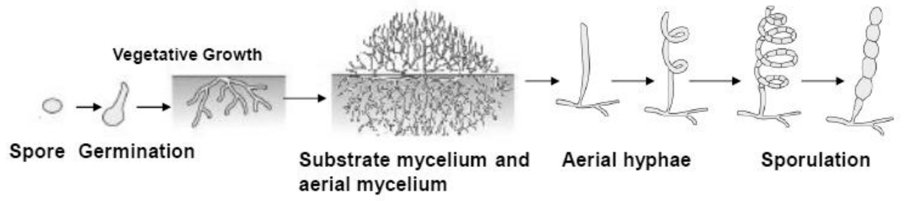


Figure 3.3: Life cycle of Actinobacteria.

(ii) Spore chain morphology: The initial steps of sporulation in several oligosporic *Actinobacteria* can be regarded as budding processes, because they satisfy the main criteria used to define budding in other bacteria (Fig. 3.4). Spores may be formed on the substrate and/or the aerial mycelium as single cells or in chains of different lengths. In other cases, spores may be harbored in special vesicles (sporangia) and endowed with flagella.

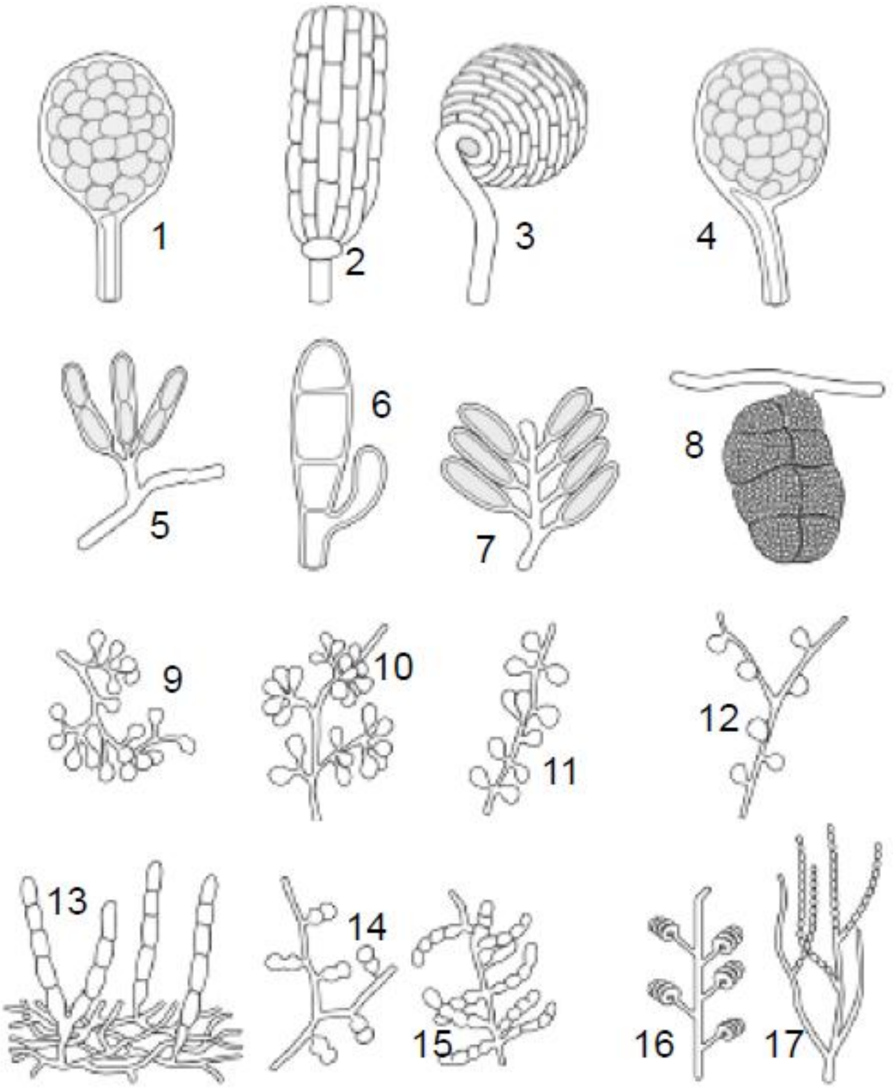


Figure 3.4: Schematic drawings of the different types of spore chains produced by actinomycetes. 1. Actinoplanes, 2. Pilimelia, 3. Spirilospora, 4. Streptosporangium, 5. Ampullariella, 6. Dactylosporangium,7. Planosmonospora, 8. Frankia, 9. Micromonospora, 10, Thermonospora, 11. Saccharomonospora, 12. Thermoactinomycetes, 13. Catellatospora, 14. Microbispora, 15. Microtetraspora, 16 &17. Streptomyces.

In the genera *Micromonospora*, *Micropolyspora*, and *Thermoactinomycètes*, spore formation occurs directly on the substrate mycelium, whereas in *Streptomyces* the spores grow out from the aerial mycelium. The *Actinoplanes* and *Actinosynnema* groups are characterized by motile spores, while *Thermoactinomyces* has unique heat-resistant endospores. Some other *Actinobacteria* genera have sclerotia (*Chainia*), synnemas (*Actinosynnem*a), vesicles that contain spores (*Frankia*), or vesicles that are devoid of spores (*Intrasporangium*). Other genera, such as *Actinoplanes*, *Ampulariella*, *Planomonospora*, *Planobispora*, *Dactylosporangium*, and *Streptosporangium*, are classified based on their sporangial morphology. (Figure 3.4) Finally, the morphology of the spores themselves can also be used to characterize species: they may have smooth, warty, spiny, hairy, or rugose surfaces.

(iii) Spore chain length. The number of spores per spore chain varies widely from genus to genus. The genera *Micromonospora*, *Salinispora*, *Thermomonospora*, *Saccharomonospora*, and *Promicromonospora* produce isolated spores, while *Microbispora* produces spores in longitudinal pairs. Members of the genera *Actinomadura*, *Saccharopolyspora*, *Sporicthya*, and some *Nocardia* spp. have short spore chains, while members of the genera *Streptomyces*, *Nocardioides*, *Kitasatospora*, *Streptoverticillium*, and some *Nocardia* spp. produce very long chains of up to 100 spores. *Frankia* species produce sporangia, which are essentially bags of spores. Streptomycetes' spore chains can be classified as being straight to flexuous (Rectus-Flexibilis), open loops (Relinaculam-Apertum), open or closed spirals (spira), or verticillate.

(iv) Melanoid pigments: Melanins like pigments black or brown and are formed by the oxidative polymerization of phenolic and indolic compounds. They are produced by a broad range of organisms, ranging from bacteria to humans. *Actinobacteria* have long beenknown to produce pigments, which may be red, yellow, orange, pink, brownish, distinct brown, greenish brown, blue, or black, depending on the strain, the medium used, and the age of the culture.

3.2.2 MATERIALS REQUIRED

1. Actinomycetes growth media
2. Actinomycetes, Streptomyces

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3. Incubator, microscope, etc

3.2.3 PROCEDURE

For the identification of actinomycetes the following experiments will be performed.

1. Observe the colour of the spore mass produced by the actinomycetes and hyphal and spore morphology.
   Inoculate the Actinomycete isolates on seven different ISP media (ISP1-ISP7) and incubate it for 5 days at 30°C. Observe the colonies under a high-power magnifying lens and note the colony morphology with respect to color, aerial and substrate mycelium, branching, and the nature of colony.
2. Gram staining procedure will be performed.
3. To perform the sugar utilization experiments in aqueous fermentation medium. Sugars such as glucose, fructose, sucrose, mannitol, rhamnose, Melibiose, xylitol at 1% will be used as carbon and energy source.
4. To perform hydrolysis experiments using starch, casein and tributyrine agars.
5. Aminoacid utilization test will be performed using the L-aminoacids such as histidine, tyrosine, cystine, phenylalanine, and valine.
6. The response of the isolate in MRVP medium, H_2S production, Urease activity and citrate functions in respective culture media will be tested.

3.2.4 OBSERVATION & RESULT

Identification of actinomycetes

Name of the test	Isolate -1	Isolate -2	Isolate -3
Spore Mass pigmentation			
Hyphal Arrangement/ Grams staining			
Spore type			
Oxidase			
Catalase			
Urease			
H_2S production			
MR (methyl Red test)			
Citrate utilization			

Carbon source utilization			
Glucose			
Fructose			
Sucrose			
Mannitol			
Rhamnose			
Melibiose			
Xylitol			
Nitrogen source utilization			
Histidine			
Tyrosine			
Cystine			
Phenylalanine			
Valine			
Hydrolytic activity			
Starch			
Casein			
Tributyrin			

RESULT: The isolated Actinomycete is identified as:

1)

2)

3)

4)

INTERPRETATION:

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3.3 LET US SUM UP

In this experiment, you have learnt about the isolation methods of antibiotic producing actinobacteria by simple crowded plate technique. Further, cross streak procedure is used for the conformation of antibiotic productivity. Identification of the Actinomycetes are performed with the morphological characters (microscopic

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examinations) along with biochemical characterization listed in the observation table (procedures given in Appendix). This experiment might be very important for screening and identification of novel antibiotic producing Actinomycetes from the soil and other environmental samples.

3.4 UNIT - END EXERCISES

1. Explain the morpologics of actinomycetes.
2. How do you isolate antibiotic producing actinobacteria? Explain.
3. Explain the identification scheme of actinomycetes.

3.5 SUGGESTED READING

1. Fakhar-un-Nisa Yunus, Zam Zam Khalid, Farzana Rashid, Asfa Ashraf1, Muhammad Naeem Iqbal, Firasat Hussain. Isolation and Screening of Antibiotic producing Bacteria from Soil in Lahore City. PSM Microbiology 2016, 1: 01-04
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UNIT – 4: PRODUCTION OF EXTRACELLULAR METABOLITES FROM ACTINOMYCETES: INDOLE-3-ACETIC ACID PRODUCTION

Structure

4.1 INTRODUCTION

The word extracellular means "outside the cell". Microorganisms produce and secrete many primary and secondary metabolites to the surrounding environment during their growth. Extracellular metabolites provide important information about the changes in microbial metabolism due to different environmental signals. Large numbers of economically valuable products are produced by microorganisms, especially actinomycetes. Actinomycetes produces, antibiotics, anti cancer, immune modulatory drugs, enzymes, siderospores, plant growth hormones etc. as extracellular metabolites.

4.1.1 Screening of IAA Producing *Actinomycetes* sp. from Soil

Indole-3-acetic acid (**IAA**, **3-IAA**) is a colorless solid, naturally occurring, plant hormone belongs to the auxin class, which directly influence the growth of the plants. IAA is a derivative of indole

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compound, with a carboxymethyl substituent soluble in polar organic solvents. IAA is mainly produced in cells of the apex and very young leaves of a plant. IAA can synthesize through four independent biosynthetic pathways starting with tryptophan as precursor. IAA has many functions inducing cell elongation, cell division, and tissue differentiation and also acts as signaling molecule necessary for development of plant organs and coordination of growth. In addition to the plant, numerous plant associated bacteria have the potentials to produce number of beneficial compounds essential for the growth of plant.

Many Rhizobacteria called plant growth promoting rhizobacteria (PGPR) are known to produce a class of phytohormones known as auxins which includes *Azospirillum, Actinomycetes, Agrobacterium tumefaciens, Bacillus, Bradyrhizobium, Enterobacter, Erwinia herbicola, Kocuria, Micrococcus, Pseudomonas,* and *Rhizobium.* Bacteria are producing the IAA from tryptophan (Trp) by different roots. Three different tryptophan–dependent production of IAA have been identified in bacteria with various intermediate compounds like IAM, IAN, ILA and IAAld. In many cases IAA can further modified into IBA and/or IPyA. Microbial IAA helps plants to produce the greatest number of root hairs resulting in increased nutrient absorption. The efficiency of these microbes can be used for the development of beneficial microbial inoculants for plant growth and management. Hence, it is important in the field of agriculture and biotechnology.

Among the various Rhizobacteria, Actinomycetes are Gram-positive saprophytic soil inhabiting bacteria. They are the most widely distributed group of microorganisms in nature. The genus Streptomyces is considered as “biological treasure” produces enormous secondary metabolites including antibiotics, anticancer, antiproliferative, anti-inflammatory, biopesticidal compounds, sederospore, IAA, etc. Several species of Streptomyces such as *Streptomyces violaceolatus, S. purpurascens, S. coelicolor, S. olivaceus, Streptomyces albus, Streptomyces himastatinicus* and *S. kasugaensis* are reported as extracellular IAA producers.

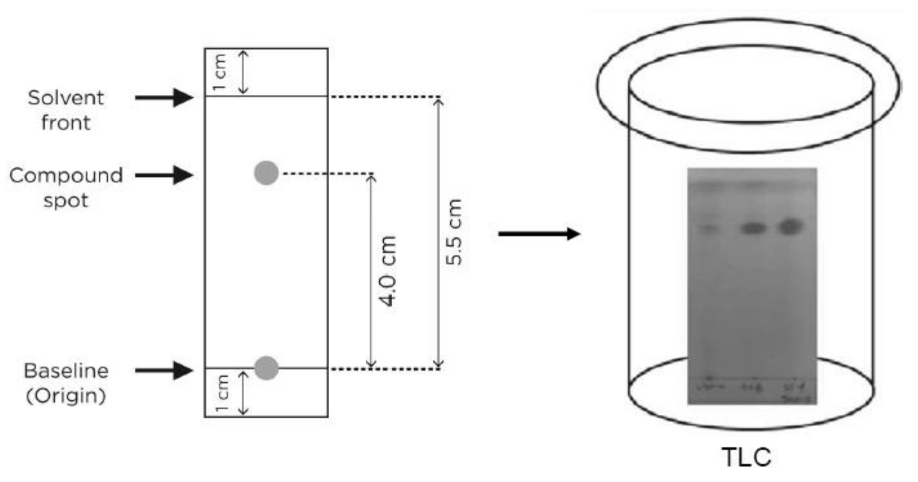
4.1.2 Conformation of IAA: Thin-Layer Chromatography

Thin-layer chromatography (TLC) is a separation technique used to separate non-volatile mixtures. Thin-layer chromatography is

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performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved. The mobile phase has different properties from the stationary phase. For example, with silica gel, a very polar substance, non-polar mobile phases such as heptanes are used. The mobile phase may be a mixture, allowing chemists to fine-tune the bulk properties of the mobile phase.

After running, the spots are visualized. Often this can be done simply by projecting ultraviolet light onto the sheet; the sheets are treated with a phosphor, and dark spots appear on the sheet where compounds absorb the light impinging on a certain area. Chemical processes can also be used to visualize spots; anisaldehyde, for example, forms colored adducts with many compounds, and sulfuric acid will char most organic compounds, leaving a dark spot on the sheet (Figure- 4.1).



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Figure – 4.1: Thin Layer Chromatography identification of IAA.

To quantify the results, the distance traveled by the substance being considered is divided by the total distance traveled by the mobile phase. (The mobile phase must not be allowed to reach the end of the stationary phase.) This ratio is called the retardation factor (R_f). In general, a substance whose structure resembles the stationary phase

will have low R_f, while one that has a similar structure to the mobile phase will have high retardation factor (Figure 4.1). Retardation factors are characteristic, but will change depending on the exact condition of the mobile and stationary phase. For this reason, known compounds are usually used to identify the unknown sample.

In this experiment, extracellular production of IAA by *Streptomyces* sp. will be performed and to conform the IAA productivity by TLC method.

2.2 OBJECTIVES

After going through this Experiment you will be able to;

- The screening methods of actinomycetes from the soil.
- To screen extracellular IAA producing actinomycetes like *Streptomycetes* sp. from soil.
- Know the assay methods of IAA.
- Understand the TLC conformation of IAA.

2.3 ABOUT THIS EXPERIMENT

In this experiment, microbial production of IAA using *Streptomyces* spp. can be performed with submerged fermentation process. The IAA production medium is supplemented with the precursor tryptophan, and the produced IAA can be assessed by Salkowski reagent method and confirmed by Thin Layer Chromatography method. Salkowski reagent reacts with indolic derivatives to develop colour. This method is simple but highly inaccurate as it gives a non-specific colour reaction with all the indolic derivatives produced by the bacteria and provides total indole content rather than specific detection of IAA. However, Thin Layer Chromatographic (TLC) gives qualitative detection of indolic compounds.

2.4 MATERIALS REQUIRED

1. Microorganism: Spectromyces *coelicolor, Streptomyces albus* (or) any of the *Streptomyces* species.
2. Media for IAA production: YM medium, Luria agar
3. Salkowsky reagent.
4. IAA standard (5 to 50µg/ml)

5. 7 Days old Streptomyces culture in tryptophan supplemented medium
6. Ethyl Acetate, HCl (1 N)
7. Silica Gel G plate
8. TLC Chamber, Sprayer
9. Instruments: Centrifuge, Spectrophotometer, cuvette, centrifuge tubes etc.

4.5 PROCEDURE

4.5.1 Screening IAA producers

1. Prepare the *Streptomyces* spp. inoculums in either LB broth or the actinomycetes broth.
2. Incubate the inoculated culture flask in 32^{0}C for 3 days in the rotary shaker.
3. Prepare the 100 ml of YM medium in 250 ml Erlenmeyer flask.
4. Add 1% log phase inoculums in to the sterile YM medium.
5. Incubate the culture at 30°C on a rotary shaking (125 rpm) for 5 days.
6. After the growth, centrifuge the content for 15 minutes at 10,000 rpm.
7. Collect the cell free supernatant for quantification of IAA.

4.5.2 Standard graph of IAA & Estimation

1. Prepare the standards of indole-3-acetic acid in acetone or ethanol ranging from 5 to 50 µg per mL of concentration (10 variations).
   Note: *IAA is least soluble in water and highly soluble in organic solvent*.
2. Take 1 ml of each indole-3-acetic acid standard in separate test tubes and add 2 ml of Salkowski reagent in each.
3. Make a blank in which use the solvent with no IAA and add the same amount of reagent.
4. Mix the reaction mixture well by gentle vortex and incubate it in the dark for 20 minutes.
5. After incubation, read the absorbance at 530 nm wavelength.
6. Plot a standard calibration graph for IAA standards by plotting a graph of absorbance versus IAA concentrations.
7. Perform the same thing for the samples containing an unknown amount of indole-3-acetic acid and the levels of IAA are determined in µg /ml.

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4.5.3 Extraction of Indolic derivatives

1. Collect the supernatant of 7 days old Streptomyces culture in YM media supplemented with Tryptophan.
2. Acidify the supernatant to pH 2.5 using 1 N HCl.
3. Add equal volume of ethyl acetate (1:1 ratio), mix thoroughly, and collect the organic phase, repeat this process thrice using equal volume of ethyl acetate.
4. Air dries the ethyl acetate fraction and redissolved in 1/10 volume of methanol.

4.5.4 Thin layer chromatography

1. Prepare silica gel G plate with calcium carbonate (or) Use commercial TLC slides.
2. Load 20µl of the IAA standard sample (0.1g/ml) and the test (extract) at 1 cm above the bottom of the slide.
3. Prepare TLC mobile phase solution using propanol and water (8:2 ratio)
4. Run the TLC plate in TLC chamber until it reach the 4/5 portion of the TLC slide.
5. Remove the TLC plate from the chamber, allow to air dry and incube 80^oC for 10 minutes.
6. Spray the Salkowski's reagent on the TLC plates for colour development.
7. Allow to dry at 45^oC for 5 minutes.
8. Observe the coloured spots, calculate the Rf value.

4.6 OBSERVATION & RESULT

4.6.1 Quantification of IAA production

Si.No	Standard Concentration of IAA (µg/ml)	Optical Density at 530nm
1	5	
2	10	
3	15	
4	20	
5	25	
6	30	

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7	35	
8	40	
9	45	
10	50	
11	Test	

4.6.2 TLC Observation

	Distance travelled by the solvent (cm)	Distance travelled by the solute (cm)	Rf value
Standard -1 (IAA)			
Standard -2 (IBA)			
Sample -1			
Sample -2			

Result

(a) The amount of IAA produced by the actinomycetes (*Streptomyces* sp.) is: ----- µg /ml.

(b) -------------------- is present in the extrcellular metabolite of the actinomycetes culture.

Interpretation

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4.7 LET US SUM UP

In this experiment, you have learnt about the isolation methods of IAA producing *Streptomyces* sp. using tryptophan supplemented medium. Further, conformation will be carried out by Thin layer Chromatography method.

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4.8 UNIT - END EXERCISES

1. Explain IAA production using *Streptomyces*.
2. State the significance of Salkowski's test.
3. Describe the TLC conformation of IAA.

4.9 SUGGESTED READING

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3. B. Mohite. Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. Journal of Soil Science and Plant Nutrition

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UNIT – 5: PRODUCTION OF INDUSTRIALLY IMPORTANT ENZYMES BY SUBMERGED FERMETATION

Structure

5.1 INTRODUCTION

Amylases are carbohydrate hydrolytic enzymes, broadly classified into α, β, and γ amylases. α-Amylase (EC 3.2.1.1) are fast active enzymes., they act on α-1-4 glycosidic bonds (glycosidic hydrolases). Further, amylases can be divided into exoamylases (hydrolyse the substrate at non-reducing end), and endoamylases (random hydrolytic at variable length). Basically, amylase substrates are obtained from plants. α-Amylases hydrolyse starch into glucose monomers and maltose disaccharides. Starch composed of amylose (linear ~ 6000 units with α-1,4 glycosidic bonds) and amylopectin (α-1,4-linked chains of 10–60 glucose units with α-1,6-linked side chains of 15–45 glucose units) with multiple industrial applications. Amylases are one which makes up approximately 25% of the world enzyme market. It used as basic raw material in foods, detergents, paper, textile and pharmaceuticals industries. In food industry, the production of

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glucose syrups, maltose syrups, corn syrups, and alcohol fermentation and baking amylases are used. Amylases are largely used in brewing industries for digesting complex polysaccharides to simple amylase and amylopectins.

Amylases are obtained from variety of microbial sources including bacteria, fungi and actinomycetes. Among the wide range of microbial species, bacteria dominate with its fast and effective enzyme production. Large number of bacteria such as Bacillus, Corynebacterium, Chromohalobacter, Geobacillus, Lysinibacillus are important genera of a-amylase producers. Among them, the genus Bacillus dominates the industrially useful amylase production. The ability of amylase production varies from one microbe to another, even among the same genus, species, and origin of the strain. They can able to produce variety of a-amylases like thermostable enzymes with high commercial value. To produce hyper active amylase production, newer isolates have been isolated from various environmental samples including soil sediments, humus, marine and estuarine sediments and hyper thermal vents. Extremophls from marine and other harsh environment produce more stable enzymes. Bacterial amylases have wide applications in brewing, backing, detergent, textile and pharmaceutical industries. Microbial amylases help to prepare maltose, and high fructose syrup. Generally, bacterial mediated enzyme production is carried out by submerged fermentation process. The major advantage of using microorganisms for production of amylases is in bulk production with low cost. The microbial amylases meet commercial demands, and it is an alternative for chemical hydrolysis of starch processing industries. Hence researchers search novel and better amylase producing strains from different sources.

To the industrial production, bacteria have been subjected under either submerged fermentation (aqueous media with starch as the substrate) or solid state fermentation (intact natural starch rich substrate supplement with minimum amount of growth requirements). For the large scale operation different methods have been established for enzyme production.

5.2 OBJECTIVES

After going through this Experiment you will be able;

- To screen the amylase producing bacteria from the soil.

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- To produce extracellular amylase by submerged fermentation.
- To know the assay methods of amylase enzymes.

5.3 ABOUT THIS EXPERIMENT

5.3.1 Screening α-amylase producers

The isolation of microorganism is potentially important before being screened for their production of enzymes of interest. Amylase producing microbes are ubiquitous and are usually obtained from soil sediments. Two methods are generally useful for screening of α-amylase producers; they are 1) solid-media based, or 2) aqueous media-based techniques. The common method is standard plating technique on the solid media with starch as the substrate. The second method (substrate selection) is through enrichment before screening of the isolate on solid media, where efficient strains are isolated according to their affinity for a particular substrate. Through these methods, different bacteria and fungi have been screened and studied for amylase production.

In this experiment, serial dilution based method can be used to screen the amylase producing bacteria. Subsequently the enzyme production is quantified based on the amount of resulting end product produced by the dinitro salicylic acid (DNS) and Nelson-Somogyi (NS) techniques. In the solid-agar method, the appropriate strain (bacteria) is inoculated onto the starch-containing agar at the center of the Petri plate. After an appropriate incubation period, the plate is flooded with iodine solution, which reveals a dark bluish color on the substrate region (starch-iodine complex) and a clear region (due to hydrolysis) around the inoculum, indicating the utilization of starch by the microbial amylase. Further, pure culture of large hydrolytic zone producing bacteria will be grow in aqueous media with starch (1 to 2% w/v), subsequently total enzyme productivity will be quantified by DNS based method. In this method enzyme and the starch substrate are mixed and incubated for 10 min at 50°C. Then, cool to room temperature, the absorbance of the solution is read at 540 nm. This method is to detect the release of reducing sugars from substrate hydrolysis by amylase enzyme.

5.3.2 Submerged Fermentation

In this method mineral media or the nutrient media supplemented with high starch substrate is used. Bacteria growing in submerged

fermentation produce extracellular amylases and depolymerize the starch in to simple sugars for its growth and energy requirements. The amount of enzyme production will be quantified and the productive capacity of the organism can be determined. In this experiment, culture supernatant can be used as enzyme source, using this reaction will be performed with standard starch solution at a standard time period. During this reaction enzyme react on the starch substrate to produce maltose sugars, described below.

$$\text{Starch} + H_2O \xrightarrow{\alpha\text{-Amylase}} \text{Reducing Sugar (Maltose)}$$

Amylase activity can be estimated by the analysis of reducing sugar (maltose) released during hydrolysis of 1% (w/v) starch. The amount of maltose is estimated by 3,5-dinitrosalicylic acid method. One unit of amylase activity is defined as the amount of enzyme that release 1mMol of reducing sugar as glucose per min under assay conditions. Enzyme activity is expressed as the specific activity, which represents as U/ml.

DNS method Principle: Reducing sugars have the property to reduce many of the reagents. One such reagent is 3,5-dinitrosalicylic acid (DNS). 3,5-DNS in alkaline solution is reduced to 3 amino 5 nitro salicylic acid.



3,5-dinitrosalicylic acid (yellow) — 3 amino, 5-nitro salicylic acid (orange-red)

Figure – 5.1: Dinitro saliculic acid.

5.1.4 MATERIALS REQUIRED

1. Media: Starch agar, starch broth, nutrient agar, nutrient broth
2. Reagents: Iodine solution.
3. Sample: Soli sediment.
4. Others: L-rod, Laminar hood, burner, pipettes, test tubes with sterile water blanks, etc
5. Microorganism: Pure culture of *Bacillus amyloliquifaciens* (or) *B. licheniformis* (or) *Bacillus subtilis.*
6. Buffer: 0.02 M sodium phosphate buffer (pH, 6.9) with 0.006 M sodium chloride.

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7. Starch solution: 1.0% starch solution in 0.02 M sodium phosphate buffer (pH, 6.9).
8. Maltose stock solution: 1mg/ml, stored at 4^0C.
9. Reagents: DNS reagent
10. Others: Laminar hood, burner, pipettes, Erlenmeyer flask, Shaking incubator, test tubes, sterile water blanks, Spectrophotometer, cuvettes, etc.

5.5 PROCEDURE

5.5.1 Screening α-amylase producers

1. Prepare starch agar plates.
   a. Prepare ten starch agar plates (sterile) with 1% starch as carbon and energy source.
   b. Mark the media plates and keep this in the inoculation chamber.
2. Sample preparation and serial dilution.
   a. Take one gram of soil in to a 10 ml of sterile water blank. Vortex the content and allow to settle down for the soil particles.
   b. Perform a serial dilution procedure to transfer one ml of the soil suspension to the second tube and mix thoroughly.
   c. Repeat the step up to 10^{-7} dilutions.
3. Plating the sample
   a. Take 0.1 ml of the diluted soil samples (10^{-4} to 10^{-7}) and inoculate on the sterile starch media.
   b. Using L-rod to spread the sample at the entire surface of the media.
   c. Incubate the plates at 32 to 37^{o}C for 48 hours.
   d. Observe the growth of the bacteria after the incubation period.
4. Observe the results.
   a. After the bacterial growth the agar surface is flooded with iodine solution for 30sec. decant the excess iodine and observe the clear transparent zone around the bacterial colony, with dark blue background.
   b. Count the total number of colonies and the starch hydrolytic colonies.
   c. Measure the zone of starch clearance with standard ruler.
   d. Select any five to ten effective colonies and maintain as pure culture.

5.5.2 Submerged fermentation of α-Amylase

1. Bacterial inoculum is prepare in nutrient broth inoculated with a loopfull of bacteria from the mother slant and incubate it for 24 hours to reach log phase growth (10^8 to 10^9 cfu/ml).
2. Prepare 100 ml of media in 250 ml Erlenmeyer flask for amylase fermentation and sterilize it [a nutrient medium supplement with 1% (w/v) starch as carbon and energy source].
3. Aseptically transfer 1.0 ml of log phase inoculums to the fermentation medium.
4. Incubate the flask for 24 to 48 hours at incubated shaker (35^0C).
5. After incubation, centrifuge the fermented media at 8000 rpm for 10 min.
6. Transfer the supernatant to the fresh tube for enzyme assay.

5.5.3 α-Amylase Enzyme assay

1. Preparation of the D.N.S. reagent: 10 g of 3,5-dinitrosalicylic acid are gradually dissolved under heating conditions, in 700 ml of NaOH solution 0.5 N. Then 300 g of sodium potassium tartrate ($NaKC_4O_6.4H_2O$) are added and distillated water was added in the mixture until a final volume of 1000 ml. The reagent has a dark orange colour and is stable for several days in room temperature.
2. Prepare standard graph for maltose as follows:
   a. Standard solutions of maltose (0-10 μmoles/l) are prepared in test tubes.
   b. 1 ml of D.N.S. reagent is added in each tube and the mixture is agitated for a few seconds on vortex mixer.
   c. The samples are placed in a water bath (T=100°C) for 5 min and then they are left to cool at room temperature.
   d. 5 ml of deionized water are added in each sample, followed by agitation.
   e. The absorbance (A) of the samples is measured at λ=540 nm.
   f. A standard curve is being drawn.
3. Enzyme reaction is performed in a test tube described below;
   a. Pipette out 0.5 mL enzyme solution (step-1 6th point) and incubate tubes at 25 ^oC for 3 min.
   b. Add 0.5 mL starch solution and incubate for 5 min (Room Temperature).
   c. Stop the reaction by adding 1 mL DNS reagent.
   d. Heat the solution in a boiling water bath for 5 min.
   e. Cool it in running tap water.

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f. Make up the volume to 10.0 mL by the addition of distilled H_2O.
g. Blank is prepare without enzyme, and follow the above steps.
h. Read the absorbance at 540 nm using UV-Vis Spectrophotometer.

5.6 OBSERVATION & RESULT

5.6.1 Qualitative determination of α-amylase producer

Si. No	Dilution factor	Plate name	Number of colonies [CFU]		% of starch hydrolytic colonies
			Total CFU	Starch hydrolysis CFU	
1	10^{-5}				
2	10^{-6}				
3	10^{-7}				

Calculation: % Starch hydolytic organisms = Number of CFU with clear zone x 100 /Total number of colonies

Note:
Positive result: *Following bacterial inoculation and incubation, bacterial colony developed with colourless clear hydrolytic zone.*
Negative result: *The bacterial colony without clearing zone*

5.6.2 Sugar Standard Graph

Si. No	Concentration of Glucose (mg/ml)	OD at 540 nm	Si. No	Concentration of Glucose (mg/ml)	D at 540 nm
1	0.1		8	0.8	
2	0.2		9	0.9	
3	0.3		10	1.0	
4	0.4		E-B	--	
5	0.5		S-B		
6	0.6		Test		
7	0.7		Test		

Calculation of α-amylase activity

One unit (U/mL) of α-amylase activity is defined as: the amount of protein (α-amylase) required to liberate 1 µmol (0.18 mg

equivalence) of reducing sugar (D-glucose) from starch/min, under the assay conditions.

$$\text{Formula: } \alpha\text{-amylase activity (U/mL)} = \frac{\Delta E \times Vf}{\Delta t \times \sum \times Vs \times d}$$

ΔE = Absorbance at 540 nm

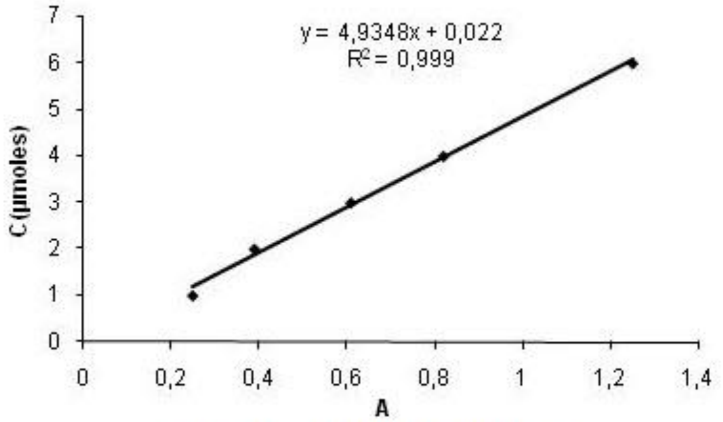
Vf = Final volume including DNS

Vs = Volume (mL) of α-amylase used

Δt = Time of hydrolysis

$\sum$ = extinction coefficient = 0.165 cm^2/µm glucose

d = diameter of the cuvette = 1



Standard curve for maltose

Result: The amount of enzyme produced by the bacteria at the give time period is = ______ U/ml.

Interpretation

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5.7 LET US SUM UP

In this experiment, you have learnt about the isolation methods of amylase an extracellular polysaccharide hydrolytic enzyme producing bacterial species using starch agar medium following with α-

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amylase production and estimation. The reducing sugar analysis also learnt through this method.

5.8 UNIT - END EXERCISES

1. Explain amylases producing bacteria from the soil.
2. State the significance of DNS method.
3. How starch hydrolysis is visualized?

5.5 SUGGESTED READING

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UNIT – 6: PRODUCTION OF INDUSTRIALLY IMPORTANT ENZYMES BY SOLID STATE FERMENTATION

Structure

6.1 INTRODUCTION

Solid-state fermentation (SSF) is a process whereby an insoluble substrate is fermented with sufficient moisture but without free water. This process facilitate "simultaneous saccharification and fermentation, hence it become abbreviated SSF. But, SSF is commonly used for "solid-state fermentation", and it is an antonym to "liquid-state fermentation" [LSF]. In liquid-state fermentation (LSF), the substrate is solubilized or suspended as fine particles in a large volume of water are called submerged fermentation (SMF). SSF requires no complex controls and has many advantages over SMF based on its own inherent properties. This process consists of depositing a solid culture substrate, such as rice or wheat bran, beet pulp on flatbeds after seeding it with microorganisms; the substrate is then left in a temperature-controlled room for several days.

Solid state fermentation uses culture substrates with low water levels (low water activity), which is particularly suitable for mould. The solid state fermentation method used to grow filamentous fungi to allow them to reproduce in larger volume like their natural environment. The medium is saturated with water but little of it is free-flowing. The solid medium comprises both the substrate and the solid support on which the fermentation takes place. In nature, filamentous fungi grow on the ground, decomposing vegetal compounds under naturally ventilated conditions. Therefore, solid state fermentation enables the optimal development of filamentous fungi, allowing the mycelium to spread on the surface of solid compounds among which air can flow.

At the beginning of the growth process, the substrates and solid culture compounds are non-soluble composed of very large, biochemically complex molecules that the fungus will cut off to get essential C and N nutrients. To develop its natural substrate, the fungi set forth its entire genetic potential to produce the metabolites necessary for its growth. The composition of the growth medium guides the microbial metabolism towards the production of enzymes that release bio-available single molecules such as sugars or amino acids by carving out macromolecules. Therefore, when selecting the components of the growth medium it is possible to guide the cells towards the production of the desired metabolite(s), mainly enzymes that transform polymers (cellulose, hemicellulose, pectins, proteins) into single moieties in a very efficient and cost-effective manner. Compared to submerged fermentation processes, solid state fermentation is more cost-effective: require smaller vessels, lower water consumption, reduced wastewater treatment costs and lower energy consumption. In order to maintain sufficient moisture content for the growth of filamentous fungus, waterlogged air is used and may require further addition of water. In most cases, solid state fermentation does not require a completely sterile environment as the initial sterilization of the fermentation substrate associated with the rapid colonization of the substrate by the microorganism limits the development of the autochthonous flora.

There are several enzymes and industrially valuable products can be produced via SSF method. In this experiment, SSF of cellulase is described below;

Cellulose is the most abundant organic polymer on Earth. Cellulose is a polysaccharide consisting of a linear chain of several

hundred to many thousands of β(1→4) linked D-glucose units (Fig.6.1). Cellulose is an important structural component of the primary cell wall of green plants, many forms of algae and the fungi oomycetes. Some species of bacteria secrete it to form biofilms. Cellulose is mainly used to produce paperboard and paper. Smaller quantities are converted into a wide variety of derivative products such as cellophane and rayon. Conversion of cellulose from energy crops into biofuels such as cellulosic ethanol is under development as a renewable fuel source. Cellulose for industrial use is mainly obtained from wood pulp and cotton

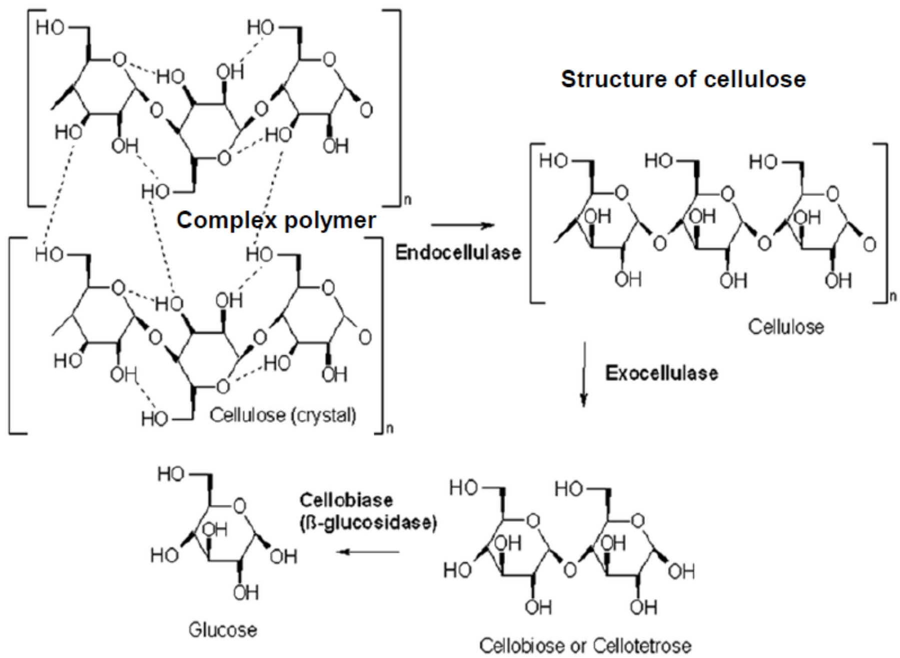


Fig-6.1: Structure of cellulose and its hydrolysis.

Cellulase Enzyme

Cellulases are hydrolytic enzymes acts to hydrolyze cellulose into glucose units or oligosaccharides. It is a complex enzyme containing exoglucanase, endoglucanase, and beta-glucosidase. Presence of multiple subunits in cellulases synergistically hydrolyzes the cellulosic substrate (Fig. 6.1). Cellulases have been commercially available for more than 4 decade, and it has various biotechnological potential including food, animal feed, brewing and wine making, agriculture, biomass refining, pulp and paper, brewing, textile, and laundry as well as significantly in ethanol production. Cellulases are inducible enzymes. A large group of microorganisms including both fungi and bacteria hydrolyse cellulosic materials for its energy

requirements. Among the various groups the genera such as *Clostridium*, *Cellulomonas*, *Thermomonospora*, *Trichoderma*, and *Aspergillus* are the most extensively studied cellulase producer. Comparatively, fungal cellulases are simpler than bacterial cellulases. Fungi can produce large amount of enzymes than bacteria and it has safe applicability in industries. The species such as *Aspergillus niger* and *Tichoderma reesei* are the most important fungi produces cellulase under solid-state fermentation (SSF). SSF is economical than the submerged fermentation, which includes low investment costs for equipment and operations, high volumetric productivity, higher final product concentrations, higher product stability, lower space requirements, and easier downstream operations.

6.2 OBJECTIVES

After going through this Experiment you will be able to understand;

- To know the process of media for solid state fermentation.
- To understand the method for extraction of enzymes from solid state fermentation.
- To know the assay methods of cellulase enzymes.

6.3 ABOUT THIS EXPERIMENT

In this experiment, solid state fermentation of fungal cellulase production using *Aspergillus niger* on rice brawn medium. After the fermentation, the enzyme from the solid substrate could be recovered and estimated by standard cellulase assay (FPase) methods.

FPase is the most common total cellulase activity assay recommended by the International Union of Pure and Applied Chemistry (IUPAC). This assay is based on a fixed degree of conversion of substrate, i.e. a fixed amount (2 mg) of glucose (based on reducing sugars measured by the DNS assay) released from 50 mg of filter paper (i.e., both amorphous and crystalline fractions of the substrate are hydrolyzed) within a fixed time (i.e., 60 min). Total cellulase activity is described in terms of "filter-paper units" (FPU) per milliliter of original (undiluted) enzyme solution. The strengths of this assay are that (1) the substrate is widely available and (2) the substrate is reasonably susceptible to cellulase activity.

Endo-β-1,4-D-glucanase (EC 3.2.1.4) randomly cleaves available intermolecular β-1,4-glucosidic bonds on the surface of

cellulose. Because insoluble cellulose has very low accessible fractionation of β-glucosidase bonds to cellulase, water-soluble cellulose derivatives such as CMC and hydroxyethylcellulose (HEC) are commonly used for endoglucanase activity assay. The hydrolysis can be determined by measuring the changes in reducing sugars or viscosity or color. Since CMC is an anionic substrate, its properties change with pH. The reducing sugar concentration is measured by the DNS method.

6.4 MATERIALS REQUIRED

1. Microorganism: *Aspergillus niger*, *Tricoderma* sp.
2. Substrate for SSF: Rice bran, rice husk, and rice straw will be used.
3. Minimal Salt Solution.
4. Others: Conical flask, Incubator, etc.
5. DNS (3,5-dinitrosalicylic acid) reagent.
6. Citrate buffer (1 M, pH 4.8).
7. Filter paper strip (50 mg, 1.0 × 6.0 cm): Cut 1.0 × 6.0 cm Whatman No. 1 paper strips with a paper cutter.
8. Glucose standard stock solution – 10 g/l. Citrate buffer (50 mM, pH 4.8)
9. CMC (2% w/v) in citrate buffer

6.5 PROCEDURE

6.5.1 Preparation of Spore suspension

1. Spore of these fungal cultures will be grown on the Potato Dextrose Agar media for 24 hours.
2. The spores will be harvested by gently add sterile saline water to the agar plate surface containing spores and scrap.
3. Collect the water along with spores in a fresh sterile tube. Centrifuge at 8000 rpm for 10 minutes.
4. Collect the spore pellet redissolved in sterile saline and centrifuged to prepare fungal spore suspension. The final spore suspension will be adjusted based on the inoculums level.

6.5.2 Substrate for SSF

1. Different substrates from the by-products of rice such as rice bran, rice husk, and rice straw will be used.

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2. 10g of substrate is taken in250-ml flask and add sufficient quantity of water with mineral supplements (1ml) to the substrate.
3. Autoclave the media at121°C for 20 min.
4. After cooling to room temperature, 1 ml of spore suspension [5 x 10^7 spores/ml].
5. Incubate the flask at room temperature for 48 to 72 hours.

6.5.3 Enzyme extraction

1. Cellulase enzyme will be extracted by distilled water extraction method.
2. After incubation, the weight of the fermented solid substrate will be determined.
3. Add five fold volume of distilled water. Stir for 30 min at 700 rpm.
4. Collect the media suspension in a centrifuge tubes and the biomass as well as the residual substrates will be removed by centrifugation at 8000 rpm for 15 min.
5. Collect the supernatant – as cellulase enzyme.

6.5.4 Filter Paper (FPase) Enzyme assay

1. Place a rolled filter paper strip (1 x cm size) into each 13 × 100 test tube.
2. Add 1.0 ml of 50 mM citrate buffer (pH 4.8) to the tubes; the paper strip should be submerged in the buffer.
3. Prepare the enzyme dilution series, of which at least two dilutions must be made of each enzyme samples.
4. Prepare the dilute glucose standards as 0, 2, 4, 8 … 20 mg/ml in separate tubes [0.5ml /tube].
5. Add 1.0 ml of 0.050 M citrate buffer.
6. Prepare the blank and controls.
7. Reagent blank (RB): 1.5 ml of 50 mM citrate buffer.
8. Enzyme controls (EC): 1.0 ml of 50 mM citrate buffer + 0.5 ml enzyme source
9. Substrate control (SC): 1.5 ml of 50 mM citrate buffer + filter paper strip.
10. Test enzyme (TE-1-2): dilution series whose enzyme concentrations are the same as those from E1 to E5 (seeNote7).
11. Prewarm the enzyme solutions, blank, and controls until equilibrium.

12. Add 0.5 ml of the enzyme dilution series to the tubes with filter paper substrate (TE1–2); add 0.5 ml of the enzyme dilution series to the tubes without filter paper substrate (EC).
13. Incubate the tubes of in a 50°C water bath for exactly 60 min.
14. Add 3.0 ml of the DNS reagent to stop the reaction, and mix well.
15. Boil all tubes for exactly 5.0 min.
16. Transfer the tubes to an ice-cold water bath.
17. Withdraw ~0.5 ml of the colored solutions into 1.5-ml micro-centrifuge tubes and centrifuge at ~10,000 g for 3 min.
18. Add 0.200 ml of the supernatant into 3-ml spectrophotometer cuvette tubes, add 2.5 ml of water, and mix well by using a pipette or by inversion several times.
19. Measure absorbance at 540 nm, where the absorbance of RB is used as the blank.
20. Draw a standard sugar curve (sugar along the x-axis vs. absorbance at 540 along the y-axis).

Calculate the FPase of the original concentrated enzyme solution in terms of FPU/ml:

$$= \frac{0.37}{\text{enzyme concentration to release 2.0 mg glucose}} \text{ units ml}^{-1}$$

6.5.5 Endo-β-1,4-D-glucanase (CMCase)

1 Prepare the enzyme solution, of which at least two dilutions must be made of each enzyme sample [one dilution releasing slightly more than 0.5 mg of glucose and one slightly less than 0.5 mg of glucose].
2 Add 0.5 ml of the enzyme source into test tubes with a volume of at least 25 ml.
3 Equilibrate the enzyme solution and substrate solution at 50°C.
4 Add 0.5 ml of the CMC solution to the test tubes and mix well.
5 Incubate at 50°C for 30 min.
6 Stop the reaction with add 3.0 ml of DNS solution and mix well.
7 Boil for exactly 5.0 min in boiling water.
8 Place the tubes in an ice-cooled water bath to quench the reaction.
9 Add 20 ml of distilled water and seal with parafilm or by a similar method. Mix by inverting the tubes several times.
10 Read the absorbance at 540 nm based on the substrate blank.
11 Prepare a) substrate blank (0.5 ml of CMC solution + 0.5 ml of citrate buffer), b) enzyme blanks (0.5 ml of CMC solution + 0.5

ml of dilute enzyme solutions), treat substrate and enzyme blanks similarly.

12 Prepare the glucose standards: 0.1, 0.2, 0.3 ….1.0mg/ml in citrate buffer. Follow the steps 6 to 10.

13 Plot the standard graph. Calculate the glucose released by the enzyme solutions with deduction of the enzyme blank absorbance based on the glucose standard curve.

14 Calculate the CMCase activity of the original concentrated enzyme solution in terms of IU/ml:

$$\text{CMCase} = \frac{0.185}{\text{EDR}}$$

6.6 OBSERVATION & RESULT

6.6.1 Qualitative determination of FPase producer

Si. No	**Concentration of Glucose (mg/ml)**	**OD at 540 nm**	**Si. No**	**Concentration of Glucose (mg/ml)**	**OD at 540 nm**
1	0.1		**8**		
2	0.2		**9**		
3	0.3		**10**		
4	0.4				
5	0.5				
6	0.6				
7	0.7				

6.6.2 Observation: Sugar Standard Graph (CMCase)

Si. No	Concentration of Glucose (mg/ml)	OD at 540 nm	Si. No	Concentration of Glucose (mg/ml)	D at 540 nm
1	0.1		8	0.8	
2	0.2		9	0.9	
3	0.3		10	1.0	
4	0.4		E-B	--	
5	0.5		S-B		
6	0.6		Test		
7	0.7		Test		

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Result

a) The FPase activity of the original concentrated enzyme solution in terms of IU/ml: ------------.

b) The CMCase activity of the original concentrated enzyme solution in terms of IU/ml: ------------.

Interpretation

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6.7 LET US SUM UP

In this experiment, you have learnt about the solid state fermentation process, media preparation, extraction of enzymes from the solid state fermented media, and the enzyme assay. In this experiment, Filter Paper hydrolase (FPase) and Carboxymethyl cellulase (CMCase) assay methods has been described. The reducing sugar analysis also learnt through this method.

6.8 UNIT - END EXERCISES

1. Explain solid state fermentation process.
2. State the significance of cellulase.
3. How the enzymes extracted from the SSF media?
4. Demonstrate the CMCase aassay.

6.9 SUGGESTED READING

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UNIT – 7: ASSAY OF EXTRACELLULAR ENZYMES

Structure

7.1 EXTRACELLULAR ENZYMES

Extracellular enzymes have lot of industrial applications. Many microorganisms have been used as a source for industrial production of enzymes. To knowing the proper methods used for determination and quantification of the respective enzyme is most important in industrial large scale fermentation of enzymes. Usually, culture supernatant of the submerged fermentation or the aqueous extract of the solid state fermentation is the source for extracellular enzymes. Mostly, spectrophotometric (colorimetric), titrimetric, and gel diffusion assay procedures are used. They are easy and simple detections methods and could be perform any of the basic laboratories. Using suitable reaction procedure followed with the quantification of the residual substrate

concentration or the end product quantity will help to calculate the total concentration of enzymes (IU) present in the enzyme source. Different colouring materials (chromogen or flurochromes) have been used to determine the concentration of the direct end product or the reaction or the co-metbolite of the reaction. The assay procedure must be reaction sensitive and should be ideal for quantification of the least concentration of enzymes. In this chapter, you are going to perform the assay of three most important industrial enzymes such as, 1) α-amylase, 2) alkaline protease, and 3) lipase.

7.2 OBJECTIVES

After going through this Experiment you will be able to understand;

- The method of α-amylase enzyme.
- The method for alkaline protease assay.
- The assay methods of lipase enzymes.

7.3 AMYLASE ENZYME ASSAY

7.3.1 Principle

Amylases are enzymes that break down starch or glycogen. The amylases can be derived from several sources such as plants, animals and microbes. In this experiment, α-amylase enzyme reaction will be performed with standard starch solution at a standard time period. During this reaction enzyme react on the starch substrate to produce maltose sugars, described below.

$$\text{Starch} + H_2O \xrightarrow{\alpha\text{-amylase}} \text{Reducing Groups (Maltose)}$$

Amylase activity can be estimated by the analysis of reducing sugar (maltose) released during hydrolysis of 1% (w/v) starch. The amount of maltose is estimated by 3,5-dinitrosalicylic acid method. One unit of amylase activity is defined as the amount of enzyme that release 1mMol of reducing sugar as glucose per min under assay conditions. Enzyme activity is expressed as the specific activity, which represents as U/ml.

DNS method Principle: Reducing sugars have the property to reduce many of the reagents. One such reagent is 3,5-dinitrosalicylic acid

NOTES

(DNS). 3,5-DNS in alkaline solution is reduced to 3 amino 5 nitro salicylic acid.



7.4.1 MATERIALS REQUIRED

1. Buffer: 0.02 M sodium phosphate buffer (pH, 6.9)
2. Enzyme source: Culture supernatent
3. Starch solution: 1.0% in 0.02 M sodium phosphate buffer (pH, 6.9).
4. Maltose stock solution: 50mg/ml.
5. Reagents: DNS reagent.
6. Others: Pipettes, Erlenmeyer flask, test tubes, sterile water blanks, Spectrophotometer, cuvettes, etc.

7.4.3 PROCEDURE

1. Prepare standard graph for maltose as follows:
   a. Standard solutions of maltose (0-10 µmoles/l) are prepared in test tubes.
   b. 1 ml of D.N.S. reagent is added in each tube and the mixture is agitated for a few seconds on vortex mixer.
   c. The samples are placed in a water bath (T=100°C) for 5 min and then they are left to cool at room temperature.
   d. 5 ml of deionized water is added in each sample, followed by agitation.
   e. The absorbance (A) of the samples is measured at λ=540 nm.
   f. A standard curve is being drawn.

2. Amylase Enzyme reaction
   a. Pipette out 0.5 mL enzyme solution (step-1 6th point) and incubate tubes at 25 ^{o}C for 3 min.
   b. Add 0.5 mL starch solution and incubate for 5 min (Room Temperature).
   c. Stop the reaction by adding 1 mL DNS reagent.
   d. Heat the solution in a boiling water bath for 5 min.
   e. Cool it in running tap water.
   f. Make up the volume to 10.0 mL by the addition of distilled H_2O.

g. Blank is prepare without enzyme, and follow the above steps.
h. Read the absorbance at 540 nm using UV-Vis Spectrophotometer.

7.4.4 OBSERVATION & RESULT

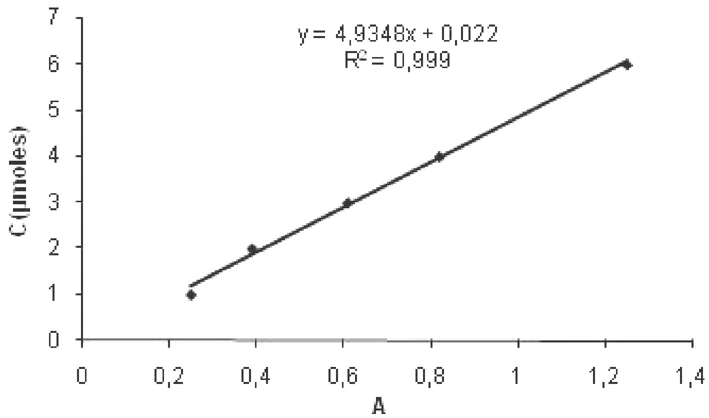
Qualitative determination of amylase enzyme

Si. No	Concentration of Glucose (mg/ml)	OD at 540 nm	Si. No	Concentration of Glucose (mg/ml)	OD at 540 nm
1	0.1		8		
2	0.2		9		
3	0.3		10		
4	0.4				
5	0.5				
6	0.6				
7	0.7				

Calculation of α-amylase activity

One unit (U/mL) of α-amylase activity is defined as: the amount of protein (α-amylase) required to liberate 1 µmol (0.18 mg equivalence) of reducing sugar (D-glucose) from starch/min, under the assay conditions.

Formula: $\alpha\text{-amylase activity (U/mL)} = \dfrac{\Delta E \times Vf}{\Delta t \times \Sigma \times Vs \times d}$



Standard curve for maltose

ΔE = Absorbance at 540 nm

Vf = Final volume including DNS

NOTES

Vs = Volume (mL) of α-amylase used
Δt = Time of hydrolysis
$\sum$ = extinction coefficient = 0.165 $cm^2/\mu m$ glucose
d = diameter of the cuvette = 1

Result: The amount of enzyme produced by the bacteria at the give time period is = ______ U/ml.

7.5 PROTEASE ACTIVITY ASSAY

7.4.1 Principle

7.4.1.1 Casein as Substrate

Proteases break peptide bonds. It is often necessary to measure and/or compare the activity of proteases. This non-specific protease activity assay may be used as a standardized procedure to determine the activity of proteases for quality control purposes. In this assay, casein acts as a substrate. When the protease digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin & Ciocalteus Phenol, or Folin's reagent primarily reacts with free tyrosine to produce a blue colored chromophore, which is quantifiable and measured as an absorbance value on the spectrophotometer. The more tyrosine that is released from casein, the more the chromophores are generated and the stronger the activity of the protease. Absorbance values generated by the activity of the protease are compared to a standard curve, which is generated by reacting known quantities of tyrosine with the F-C reagent to correlate changes in absorbance with the amount of tyrosine in micromoles. From the standard curve the activity of protease samples can be determined in terms of Units, which is the amount in micromoles of tyrosine equivalents released from casein per minute.

$$\text{Casein} + H_2O \xrightarrow{\text{Protease}} \text{Amino Acids}$$

7.5.1.2 Azoprotein Substrate

The modified and simple procedure to estimation/assay of protease enzyme is the use of an azoprotein (a protein coupled with diazotized aryl amines). The digestion of a solution with such proteins releases the chromophoric group, which is soluble in trichloroacetic acid and gives it a red-orange colour. The method itself relies on the reaction between the substrate and an enzyme under its optimum temperature/pH for a given time. The solution colour intensity is read at

440 nm, is a function of the amount of azoprotein digested, since all proteins remaining precipitate after the addition of trichloroacetic acid. The method is still one of the most reliable methods to study the proteolytic activity of enzymes due its colour stability and no need of chromogenic reagents. Besides, the sulphanilamide-azocasein's preparation is no longer necessary, since it is now available widely in the market.

7.4.2 MATERIALS REQUIRED

7.4.2.1 Casein as substrate

1. Enzyme source
2. Potassium Phosphate, buffer pH7.5.
3. Casein (0.65%) solution.
4. Trichloroacetic Acid 110 mM..
5. Folin & Ciocalteu's Phenol Reagent: 0.5 M or ½ dilutions.
6. Sodium Carbonate, Anhydrous (500 mM)
7. Sodium Acetate, Trihydrate (10 mM)
8. L-Tyrosine, Free Base (1.1 mM).
9. Equipment: Centrifuge, cetntrifuge tubes, Spectrophotometer, Cuvettes, Pipettes, Stir/Hot plate, pH Meter.

7.4.2.2 Azocasein as substrate

1. Azocasein (0.1%) in Tris-Hcl buffer (0.1M) pH 8.0
2. TCA-acetone
3. 5% trichloroacetic acid (TCA), 0.5 N NaOH
4. Spectrophotometer, cuvette
5. Centrifuge, eppendroff tube

7.4.3 PROCEDURE

7.4.3.1 Tyrosine standard graph

1. Take six test tubes and the 1.1 mM tyrosine standard stock solutions is added with the following volumes in mL: 0.05, 0.10, 0.20, 0.40, 0.50.
2. Don't add any tyrosine standard to the blank.
3. Once the tyrosine standard solution has been added, add an appropriate volume of purified water to each of the standards to bring the volume to 2 mL.
4. 5 mL of sodium carbonate is added to all tubes

5. Add 1 mL of Folin's reagent immediately afterward.
6. The test tubes are then mixed by swirling and incubated at 37 ºC for 30 minutes.
7. The absorbance is measured by a spectrophotometer using a wavelength of 660 nm.
8. Plot the values on the graph.

Volume of Tyrosine Standard	Tyrosine (µmol)
0.05	0.055
0.10	0.111
0.20	0.221
0.40	0.442
0.50	0.553
0.6	0.664

7.4.3.2 Protease Assay

1. Take four test tubes, add 5 mL of our 0.65% casein solution, and let them equilibrate in a water bath at 37°C for about 5 minutes.
2. Add 0.5 / 1.0 ml of enzyme solution to three of the test tubes, but not the blank (fourth one).
3. Mix them by swirling and incubate for 37°C for exactly 10 minutes.
4. After this 10 minute incubation, add the 5 mL of the TCA reagent to each tube to stop the reaction.
5. Then an appropriate volume of enzyme solution is added to each tube, even the blank, so that the final volume of enzyme solution in each tube is 1 mL.
6. Now incubate the solutions at 37 °C for 30 minutes.
7. After the 30 minute incubation, filter each of the test solutions and the blank using a 0.45 µm polyethersulfone syringe filter (or) centrifuge to settle down the precipitated protein at 8000 rpm for 10 minutes.
8. Take 2 ml of the filtrate / supernatant in to a new test tube
9. Add 5 mL of sodium carbonate and 1 mL of Folin's reagent is added.
10. Mixed properly by swirling and incubated at 37 ºC for 30 minutes [water bath].

NOTES

11. The absorbance is measured by a spectrophotometer using a wavelength of 660 nm.

7.4.3.3 Calculating Enzyme Activity

To get the activity of enzyme in units per/mL, perform the following calculation.

$$\text{Units/mL Enzyme} = \frac{(\mu\text{mol tyrosine equivalents released}) \times (11)}{(1) \times (10) \times (2)}$$

11 = Total volume (in milliliters) of assay

10 = Time of assay (in minutes) as per the Unit definition

1 = Volume of Enzyme (in milliliters) of enzyme used

2 = Volume (in milliliters) used in Colorimetric Determination

Note: *This protocol will enable protease activity measurements. In addition, this assay is useful for ensuring that proteases have precisely determined activity before using them for your experiments. When performing this procedure, it's important to heat both the casein and tyrosine solutions slowly because boiling will cause degradation of the protein and affect assay results. Also, it's critical to prepare different blanks for standards and for each test sample.*

7.4.3.4 Azocasin as substrate - Procedure

1. Take a serious of 2.0 ml eppendroff tubes, label it, Blank, Standard 1-3, Test 1-4.
2. Take 100µl of substrate and add 125µl of enzyme source. For blank immediately keep it in 4^{o}C for the entire assay period.
3. Incubates the tubes in 37^{o}C for 1.0 hour
4. The reaction is terminated adding 750 µL of 5% trichloroacetic acid (TCA).
5. The coagulated protein is removed by centrifugation at 2000 ×g for 10 min at room temperature.
6. The obtained supernatant is then added to a 0.5 N NaOH solution using a 1 : 1 (v/v) ratio and its absorbance is read at 440 nm.

7.4.4 OBSERVATION & RESULT

a) Observation: Tyrosine Standard Graph

Si. No	Concentration of BSA (mg/ml)	OD at 660 nm	Si. No	Concentration of BSA (mg/ml)	D at 660 nm
1	0.1		8	0.8	

2	0.2		9	0.9	
3	0.3		10	1.0	
4	0.4		E-B	--	
5	0.5		S-B		
6	0.6		Test		
7	0.7		Test		

RESULT: The protease activity of the original concentrated enzyme solution in terms of IU/ml: ------------.

b) Protease assay: Azocasein as substrate

Si.No	Enzyme (IU)	OD value at 420nm
1	S1 – 2 IU/ml	
2	S2 – 4 IU/ml	
3	S3 – 8 IU/ml	
4	S4 – 20 IU/ml	
5	Test	
6	Test	
7	Test	
8	Test	

RESULT: The amount of protease in the enzyme source = _____ IU/ml.

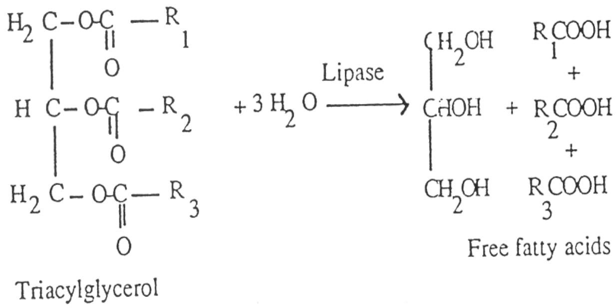
7.5 LIPASE (EC 3.1.1.3) ENZYMATIC ASSAY

7.5.1 Principle

7.5.1.1 Titrimetric Method

A common assay system for lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) activity is based on the measurement of free fatty acids, liberated from triacylglycerols, by enzymatic hydrolysis. This enzymatic reaction involves substrate concentration and the size of the surface area of the oil-water interface in the assay system. Only sufficiently dispersed and stabilized oil-water emulsions are suitable for reproducible determination of lipase activity. Stabilization of the emulsified substrate is mediated by gum arabic *(Acacia)*, which is found to be crucial to the measurement of the lipase activity in a pH-stat. The free fatty acids will be estimated by titration method with standard alkali using a suitable indicator.

Triglyceride + H_2O **Lipase** → Diglyceride + Fatty Acid



Agar Gel Diffusion Assay

Radial diffusion assay is performed by plating the sample containing the enzyme in direct contact with the substrate dissolved or suspended in agar gel. As the enzyme diffuses into the gel, the reaction takes place. At the end of a set time the gel is monitored for some estimate of enzyme activity, such as a clear area that indicates the loss of substrate. The more enzymes present in the sample, the more it diffuses from the point of application and therefore the larger the cleared zone. The clear area can be used to establish that the enzyme was indeed present in the sample and to estimate the amount present. A comparable procedure is routinely performed in assaying antibiotic concentration for. Theoretically the diffusion method can be used to determine any enzyme activity, provided that the reaction can be monitored visibly and that the substrate or product has limited diffusion through the agar gel. In practice the method has been limited to assays of hydrolytic enzymes. In this study, lipase activity can be determined by adding the sample to olive oil/ tributyrine plate. After a specific time period the presence of olive oil/ributyrine in the plate is visualized by flooding the surface with a rhodamine B /Nile-blue sulfate solution. Agar plates containing trioleoylglycerol and rhodamine B appear opaque and are pink colored. Lipase activity is monitored by irradiating plates with UV light at 350 nm. Developing an orange fluorescence zone indicates the absence of substrate and therefore the presence of the lipoytic enzyme.

7.5.2 MATERIALS REQUIRED

1. 200 mM Tris HCl Buffer, pH 7.2 at 37°C (Prepare 100 ml in deionized water using Tris Base, Adjust to pH 7.2 at 37°C with 1 M HCl)
2. Olive Oil Substrate Solution (1% v/v) in gum *Acacia.*
3. 95% Ethanol

4. 0.9% (w/v) Thymolphthalein Indicator Solution (prepare 15 ml in Reagent C using Thymolphthalein)
5. 100 ml of 50 mM Sodium Hydroxide Solution (NaOH)
6. Lipase Enzyme Solution: Freshly prepared.
7. Tributyrine agar plates in minimal media
8. Gel puncture, micropipette, tips, incubator

7.5.3 PROCEDURE

7.5.3.1 Titration Method/ Assay

1. Take 3.0mL of homogenized olive oil in (1%) gum acacia in a Erlenmeyer flask.
2. Add 1.0mL of Tris-HCl buffer pH 8.0. Mix the content thoroughly.
3. Add 2.0mL deionized water
4. Add 1.0 ml of enzyme source (at least two different concentrations)
5. The enzyme substrate mixture is incubated on magnetic stirrer at 37^{o}C for 30 minutes.
6. Keep the Blank at 0 - 4°C.
7. After 30 min of incubation, add 3.0 ml of 95% ethanol & acetone mixture (1:1).
8. Mix by swirling and then add 4 drops of thymolphthalein indicator to both the Test and Blank solutions.
9. Use a 25 ml burette with 0.1 ml graduations, titrate each solution with 0.5M NaOH to until a light blue color appear.
10. Measure the volume of NaOH for calculations

7.5.3.2 Agar Diffusion assay

1. Prepare tributyrine agar plates (or) minimal media supplemented with emulsified oil at 1% (olive oil etc.) (or) tributyrine agar plates supplemented with 2% of 0.1 **%** (w/v) Nile Blue sulphate.
2. Using gel puncture, make 6 mm or 10 mm sized wells on the tributyrine agar plates.
3. Pour variable volume of enzyme supernatant to the wells (25µl to 100µl), immediately incubate the plates at 4^{0}C for 30 min.[allow to diffuse in the gel]
4. After 30 minutes, incubate the plates at 30 to 35^{o}C in upright position until zone of hydrolysis was observed [Substrate – enzyme reaction].
5. Measure the zone diameter.

Note: Use different lipid substances to find the enzyme substrate reactions.

7.6 OBSERVATION & RESULT

a) Lipase assay- titration method

Volume of Sample taken	Test/ Assay			Blank		
	Vol. of Burette solution			Vol. of Burette solution		
	Exp-1	Exp-2	Exp-2	Exp-1	Exp-2	Exp-2
10 ml						
Final constant value						

Calculation

One unit of lipase activity was defined as one pmol of fatty acid liberated from olive oil per minute by 1.0 ml of the sample under the standard assay conditions.

$$\text{Units/ml enzyme} = \frac{\text{(A) x (Molarity of NaOH) x (1000) x (2) x (df)}}{\text{Vol. of Enzyme used}}$$

(A) = Volume (ml) of NaOH used for Test - Volume (ml) of NaOH used for Blank.

1000 = Conversion factor from ml to µl

2 = Time conversion factor from 30 minutes to 1 hour (Unit Definition)

df = Dilution factor

b) Gel Diffusion assay

Enzyme Unit	Zone diameter (mm)	Sample	Zone diameter (mm)
2 IU/ml		1	
4 IU/ml		2	
8 IU/ml		3	
10 IU/ml		4	
15IU/ml		5	
20 IU/ml		6	

RESULT

a) The amount of lipase enzyme present in the sample : _________ IU/ml (titration method).

NOTES

b) The amount of lipase enzyme present in the sample : ________ IU/ml (Agar Gel Diffusion method).

Interpretation

____

____

____

____

7.7 LET US SUM UP

In this experiment, you have learnt about the assay procedures for the extracellular enzymes like amylases, proteases and lipases.

7.8 UNIT - END EXERCISES

1. Determine the quantity of amylase enzyme.
2. Explain the method for protease assay.
3. Demonstrate the lipase assay.

7.9 SUGGESTED READING

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UNIT – 8: PURIFICATION OF ENZYMES BY FILTERATION METHOD

Structure

8.1 INTRODUCTION

Enzyme purification or protein purification are the same to purify the targeted protein of interest. One such method of purification of protein is called Gel-filtration chromatography, also known as the size exclusion chromatography. The gel filtration chromatography separates the proteins solely based on molecular size differences. In this method, a porous matrix is used to purify the protein according to their molecular size. The matrix is enclosed in a chromatographic column, and the separation is accomplished by passing an aqueous buffer through the column. The gel-filtration chromatography has numerous applications including the fractionation of proteins and other water-soluble polymers, size determination and analysis, desalting, and buffer exchange.

Gel filtration chromatography partitions molecules on the basis of molecular size and shape by means of a sieving proress. In normal molerular sieving, the gel network retards large molecules and allows small molecules to migrate through the pores. Small molecules travel farther in the gel than do large molecules. Such a pattern is observed in polyacrylamide gel electrophoresis of polynucleotides or sodium dodecylsulfate-treated proteins, in which molecules have the same charge-to-mass ratio regardless of size. In gel filtration, however, a slight but significant change in the stationary phase of causes a reversal

NOTES

in the normal order of elution. Instead of a continuous gel network, the stationary phase of gel filtration is a packed bed of porous, roughly spherical beads. The resulting channels between, the beads allow passage of large molecules. Instead of being retarded by the gel network, large molecules are excluded from the gel interior and forced to travel in the channels outside the heads. Thus, large molecules have an effectively smaller volume through which to travel than the smaller ones, and the largest molecules elute first.

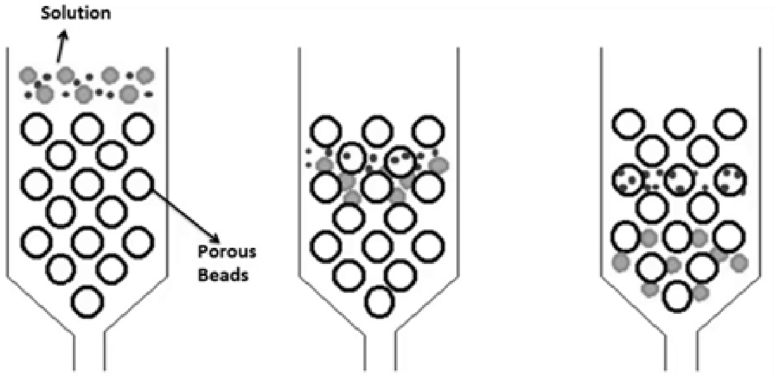


Figure – 8.1: Gel filtration chromatography.

8. 2 OBJECTIVES

After going through this Experiment you will be able to understand;

- The principles of gel-filtration chromatography.
- The method gel filtration method of protein purification.

8. 3 ABOUT THIS EXPERIMENT

In this experiment, using Sephadex G-75 gel matrix the enzyme protein will be purified. A common form of filtration bead, available under the trade name Sephadex a, is made from branched polysacrharides known as dextrans, which are composed exclusively of glucosyl residues. These dextrans are treated with the cross linking reagent epichlorohydrin to generate the porous network. Pore sue is controlled by the concentration of cross linking reagent employed.

Within the size range defined by those large molecules which are completely excluded from the gel and those small molecules which have unrestricted access the bead interior, is the fractionation range for a particular bead pore size.' Within this molecular weight range, proteins are partitioned on the basis of shape and sue according to the probability of hitting the network structure rather than passing through a

pore. This partitioning is sensitive enough to permit molecular weight determinations based on volume of eluent required to cause a protein to travel completely through the column. In general, resolution of proteins in gel filtration is affected by height and diameter of column, bead size, volume of sample and flow rate.

8.4 MATERIALS REQUIRED

1. Sephadex G-75
2. 0.05M phasphate, pH 6.9.
3. Column, stand, test-tube, etc.
4. Sample: 10mg/ml concentration of the protein in buffered (10%) sucrose solution.

8.5 PROCEDURE

8.5.1 Preparing the column

1. Place a few milliliters of the buffer into a 3 x 40 cm, coarse filtering chromatography column.
2. Pour the gel-buffer slurry into the column and allow it to pack under gravity.
3. The packed gel height is usually about 20 cm.
4. A filter paper disc is allowed to float down to the packed gel surface to minimize disturbances during sample loading.

8.5.2 Loading the Sample

1. Conventional techniques for sample loading are adequate but time-consuming.
2. Instead, apply the 1.0 ml protein sample slowly with a disposable pipette held in the column buffer after 1 cm above the gel surface.
3. The sucrose-spiked sample is significantly denser than buffer, and the protein sample can be seen layering under the buffer at the gel surface.

8.5.3 Running the Column

1. Flow rates as high as 1 to 2 ml /min can he used.
2. 2.0-ml fractions are collected. The elution profile can be evaluated visually or quantified by measuring absorbance at 380 nm for both peaks.

8.6 OBSERVATION & RESULT

Eluted protein samples will be quantified by Biuret method and verify the molecular size of the protein by SDS-PAGE method.

Table: Quantity of the protein in each fraction

Fraction No	Protein Concentration	Fraction No	Protein Concentration
F1		F6	
F2		F7	
F3		F8	
F4		F9	
F5		F10	

Result: The fraction ____ contain maximum protein of _____ µg/ml.

Interpretation

____

____

____

____

8.7 LET US SUM UP

In this experiment, you have learnt about the methods used for molecular size fractionation of the proteins and enzymes. the purity of the enzyme will be further checked with SDS-PAGE and other procedures.

8.8 UNIT - END EXERCISES

1. Explain the principles of Gel filtration method.
2. How do you get purified proteins by gel-filtration method?

8.9 SUGGESTED READING

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UNIT – 9: WINE PRODUCTION

Structure

9.1 INTRODUCTION

Wine is one of the fermented products of a fruit juice. It is one of the microbial fermentation to produce desirable value-added products of commercial importance. Wine production is an alcoholic fermentation, occurs in nature in any sugar-containing mash from fruit, berries, honey, or sap tapped from palms. Yeast is a eukaryotic microorganism (singe celled fungi), act on the sugar to convert it into alcohol and carbon dioxide.

Yeasts are the main microbial source for alcoholic fermentation. Among the 8,000 strains of microorganisms, about 9 to 10 pure strains of yeasts (*Saccharomyces cerevisiae*) are used for the fermentation of grain mashes. Each strain has its own characteristics features; however, a limited number of yeasts like *Saccharomyces ellipsoides* are used in the fermentation of wines from which ethanol is distilled. The strains used in the fermentation of grain mashes are also used in the fermentation of rum from sugarcane extracts and in beer production. Generally, yeasts function best in slightly acid medium; hence, the mash, juice, sap, or extract prepared for fermentation must be acidified. The fermentation is carried out at 24° to 29°C for 48 to 96 hours, when the mash or must is ready for distillation. The alcohol content of the fermented must is about 7 to 9 percent.

Grape wine is conceivably the most common fruit juice based alcohol. Due to its commercial value, the process is well known and

documented. The grape wine production is a simple process without need of any expensive specialized equipment. It required the basic understanding with controlled fermentation which guarantees a high quality product. It needs hygienic practice to prevent contamination of wine and spoilage. The following are major basic steps for wine production; 1) crushing the grapes to extract the juice; 2) alcoholic fermentation; 3) bulk storage and maturation of the wine in a cellar; and 4) clarification and packaging.

There are two unique types of wine made from grapes; they are 1) red wine and 2) white wine. Based on the type of grapes (white or black) and the removal of grape skin determine the type of wind. Grape wine contains number of flavor rich compounds which derived from its raw materials. Presence of tannins and its derivatives gives bitterness of the wine; usually red wine has the bitterness taste due to the skin of grapes.

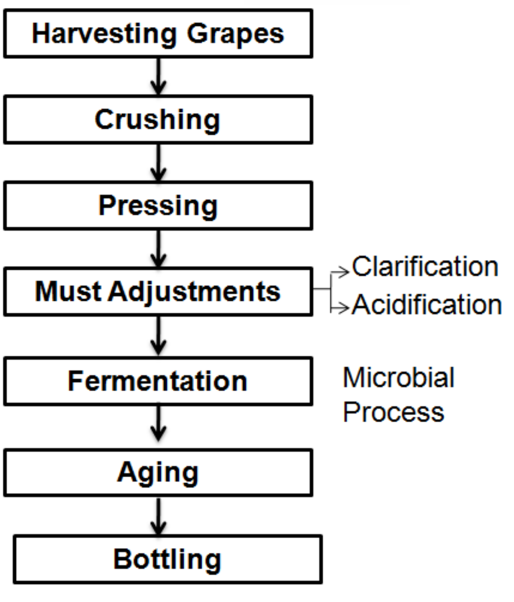


Figure -9.1: Steps involved in wine fermentation.

9. 2 OBJECTIVES

After going through this Experiment you will be able to understand;

- The process of wine fermentation.
- The wine fermentation using grape juice.
- The alcohol by spectrophotometric method.

NOTES

9. 3 ABOUT THIS EXPERIMENT

The sugars present in the fruit are fermented by yeast. When sugar is fermented by yeast, it is converted into alcohol (ethanol) and carbon dioxide gas is released. The alcohol fermentation has to take place without oxygen (anaerobic fermentation). If oxygen gets into the system during the fermentation, the alcohol will be converted into acid (vinegar (or) acetic acid). Care must be taken with the cleanliness of the equipment and personal hygiene, usually by solution of sodium or potassium metabisulphite before it is used, which can remove the contaminating microbes from the fermentation?

$$\text{Fructose of fruit syrup} \xrightarrow{\text{Yeast}} \text{Alcohol} + H_2O$$

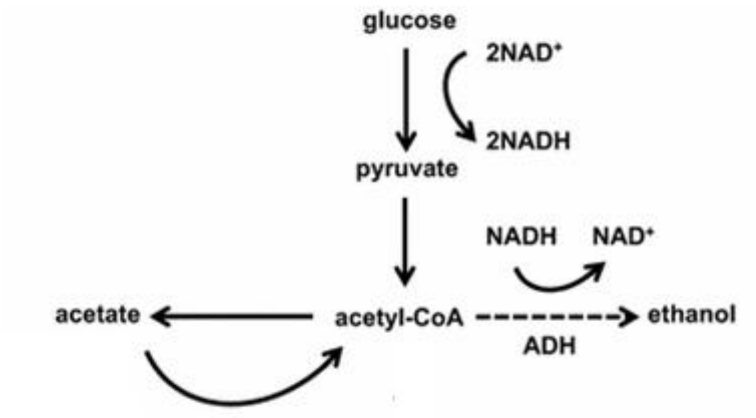


Figure - 9.2: Alcohol fermentation pathway.

Estimation of Ethanol by dichromate method

Most of the chemical oxidation methods are based on the complete oxidation of ethanol by dichromate in the presence of sulfuric acid with the formation of acetic acid. This reaction is highly preferred because potassium dichromate is easily available in high purity and the solution is indefinitely stable in air. The reaction that occurs between alcohol and potassium dichromate is:

$$2Cr_2O_7^{2-} + 3C_2H_5OH + 16H^+ \rightarrow 4Cr^{3+} + 3CH_3COOH + 11H_2O$$

Dichromate ethanol **Acetic acid**

Dichromate ($Cr_2O_7^{2-}$, Cr(VI)) is yellowish in color and the reduced chromic product (Cr^{3+}, Cr(III)) is intensely green. Because the absorption spectra of dichromate and chromic ions overlap significantly, Beer's law is not obeyed. Instead, the spectra of the

solution of interest must be analyzed at multiple wavelengths to calculate the individual concentrations of dichromate and chromic ions in a mixture subject to the material balance that the total number of chromium atoms must be conserved. Proper concentration of sulfuric acid in the surrounding solution will direct the oxidation of ethanol toward acetic acid instead of acetaldehyde.

9.4 MATERIALS REQUIRED

1. Grapes, Sugar, Wine yeast, Boiled water
2. Erlenmeyer flask, Funnel, Syphon tube, Sterilizing solution (sodium metabisulphite), Wine bottles and corks, Corking machine.
3. Red wine: red grape mashes are prepared along with its skin. It contains 10 to 14% of alcohol.
4. White wine: white grape mashes are prepare without the skin. it contains 10 to 14% of alcohol.
5. Chromic acid reagent: Dissolve 33.768 potassium dichromate, K2Cr2O7, in around 500mL of distilled water. Slowly and carefully, with stirring, add 325mL of concentrated sulphuric acid (*caution – solution will become very hot*). Addition of acid can be carried out while standing the solution vessel in an ice bath if desired. Allow the solution to cool to room temperature. Transfer volumetrically to a 1.0 L volumetric flask and make to volume with distilled water. Store in amber glass. [*Caution: Potassium Dichromate is a strong oxidising agent. Chromium (VI) compounds are toxic and are known carcinogens. Avoid skin contact, ingestion or inhalation of solid particle]s.*
6. Ethanol sample – fermented distillate.
7. Ethanol standard, Distilled water, Water bath, Ice bath

9.5 PROCEDURE

9.5.1 Wine Fermentation

1. Select good quality raw material like well ripened undamaged grapes (black or white variety). It should be sweet in taste. Perform sensory evaluation - taste, colour, aroma, appearance and flavor).
2. Surface sterilizes the fruits and the equipments using 1% sodium metabisulphite solution 3 to 6 minutes, followed by complete washing with sterile warm water.

3. Grape juice (mash) is prepared aseptically by crushing in mechanical grinding device. Transfer 400 ml of the juice into 500ml Erlenmeyer flask (sterilized).
4. Measure the sugar level in the mash. The total soluble sugars should be about 22° Brix, is equal to 22% sucrose. Adjust the pH to near slightly acidic (4 to 5) using HCl.
5. 2% inoculums of log phase yeast grow in YPD agar/ broth (~10^7 CFU/ml) is given.
6. The container (flask) is tightly closed and incubated 24 hours for aerobic growth. Then incubate it at 25^{o}C for 96 hours under anaerobic fermentation.
7. During fermentation, the yeast cells are settled down along with undigested mash precipitate. Clear solution of wine is transferred to new sterile bottle (Racking).
8. Filtrate the clear solution of wine and packed in sterile air tight bottle and store it 4^{o}C.
9. Distill the ethanol using laboratory distillation unit. Boil / warm the content at 45 to 55^{0}C, for 30 min to collect the distillate in air tight container.
10. To quantify the alcohol content in the fermented wine by acid dichromate method.

9.5.2 Alcohol Estimation

1. Prepare ethanol standard graph using standard ethanol at 1% to 10% level.
2. Collect 1 ml ethanol sample by distillation of the wine sample.
3. To 1ml of sample/solution, add 25 ml of chromic acid reagent.
4. Place the tubes in a water bath at 70ºC for 15 min.
5. Take out the tubes and immediately add 24 ml of distilled water to it to stop the reaction.
6. Measure the absorbance at 600 nm. Compare it with standard graph.

9.6 OBSERVATION & RESULT

9.6.1 Wine fermentation : Physical observation

Colour	
Smell [aroma]	
Texture	
pH	
Taste	

NOTES

9.6.2 Ethanol Standard Graph & Assay

Si.No	Concentration of Ethanol (%)	OD at 600nm
1	1	
2	2	
3	3	
4	4	
5	5	
6	6	
7	7	
8	8	
9	9	
10	10	
11	Test-1	
12	Test-1	

Plot the values in a graph. X- axis alcohol percentage and Y- axis OD value.

Result: Amount of alcohol present in the fermented grape wine is ----------.

Interpretation

9.7 LET US SUM UP

In this experiment, you have learnt about the principles and methods of wine fermentation using yeast cells. The quantification of ethanol also performed using dichromate method.

9.8 UNIT - END EXERCISES

1. Explain the protocol for wine fermentation.
2. Discuss the principles of ethanol fermentation?

3. Demonstrate the protocol for ethanol quantification.

9.9 SUGGESTED READING

NOTES

1. Nam Sun Wang. Experiment no. 12: Wine Fermentation. Department of Chemical & Biomolecular Engineering University of Maryland.
2. Cornelius S. Ough, Chemicals used in making wine, C&EN, January 5, 1987.
3. The Chemistry of Wine: Fermentation. The Oxford Companion to Wine, 4th Edition. Jancis Robinson and Julia Harding

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UNIT – 10: MICROBIAL PRODUCTION OF CITRIC ACID

Structure

10.1 INTRODUCTION

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) is an intermediate metabolite of the Kreb's cycle, it is a tricarboxylic acid contains three carboxylic functional groups with a molecular weight of 210.14 g/mol (Fig.1). Earlier, commercial production of citric acid was from imported Italian lemons (7 to 9% of citric acid) in England at 1826. Lemon juice remained the commercial source of citric acid until 1919, when the first industrial process using *Aspergillus niger* started in Belgium. Citric acid has lot of applications; hence it is one of the prime industrial products.

Citric acid is used in the production of carbonated beverages, used as a chelating and sequestering agent in the tanning and textile industry. Citrate esters are used as plasticizer. In food industries, it acts as an acidulent in the preparation of jams, preserved fruits and fruit juices, and act as a food preservative, etc. Further citric acid used in the manufacture of astringent, hair rinsers and hair setting fluids, in

beverage industry as a preservative to prevent oxidation of alcohol, and act as a emulsifier of dairy products like cheese and ice creams.

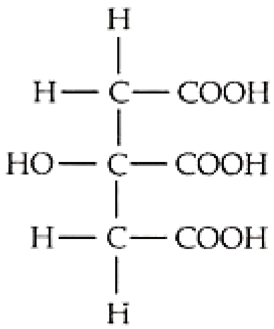


Fig.10.1. Structure of a citric acid.

Variety of microorganism especially fungi can capable of producing and accumulating citric acid in intracellular level. The fungi Aspergillus niger accumulates in greater quantities may be due to metabolic aberration. This kinds of mutated fungi can use for commercial production of citric acid. Generally, glycerol is used for making citric acid, but it is highly expensive. Microorganism such as Aspergillus wentii, A. niger, A. oryzae, A. clavatus, *Penicillium divaricatum, P. citrinum, P. luteum, Mucor pyriformis, Candida guilleirmondii, Saccharomyces lipolytica, Trichoderma viride,* and *Corynebacterium* spp. can produce citric acid. Citric acid is fermented either surface fermentation or by deep fermentation by *Aspergillus* sp. Around 60% of the industrially produced citric acid is used for various food industrial applications and in beverage industry as a flavouring agent and preservative and in pharmaceutical industry for ion-citrate.

Metabolically, *A.niger* under a deficiency of manganese, or phosphate and nitrogen, inhibits the anabolic metabolism, and the resulting degradation of proteins leads to increased ammonium ion concentration (NH_4^+). Increased concentration of glucose and NH_4^+ inhibits the function of α-ketoglutarate dehydrogenas enzyme for α-ketoglutarate production and inhibition of citric acid catabolism, therefore, citric acid is excessively accumulated (Fig. 2). The accumulation of citric acid is associated to tricarboxylate transporter activity, which competes with aconitase for citric acid. Under these conditions the affinity for citric acid is greater than that of aconitase, hence, this enzyme ejects citric acid out of the mitochondria without inhibition of enzymes of the cycle.

Various fermentation processes used for the manufacture of citric acid such as surface fermentation and submerged fermentation. In surface fermentation method either solid media or liquid media is used. Industrially, stirred thank reactor and air-lift fermenters are used. For the fermentation, *A.niger* occupies the major microbial source for industrial production of citric acid. Theoretically, 100 gram of sucrose is to produce 120g of citrate; however, 70% of the product could achieve through industrial fermentation. The previous literature shown that number of physical and chemical factors including, media pH, temperature of the fermentation, carbon, nitrogen, phosphorous sources used, agitation level and the fermenting microbes. A. niger is employed in most of the processes due to 1) easily be cultivated, 2) uniform biochemical properties, 3) large amount of citric acid with less amount of oxalic acid under controlled conditions.

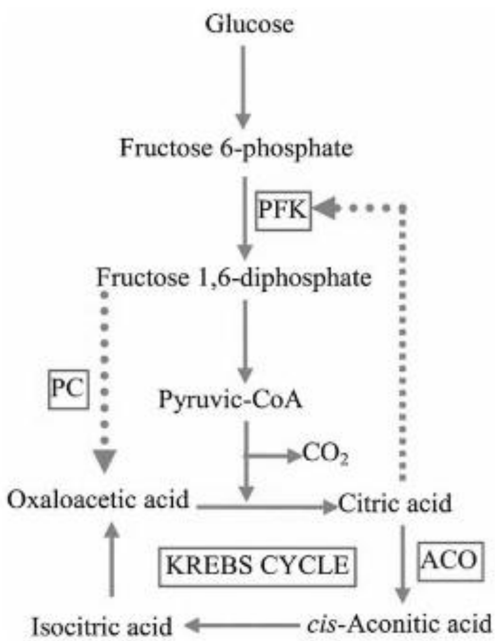


Fig-10.2: Schematic representation of the main metabolic reactions involved in the production of citric acid by *Aspergillus niger* (Soccol et al.2006).

10. 2 OBJECTIVES

After going through this Experiment you will be able to understand;

- The process of submerged fermentation of citric acid fermentation.
- The method of titration and analyze the uncertainties associated with titrations;

- Observe an acid-base reaction, and simple spectrophotometric analysis

10. 3 ABOUT THIS EXPERIMENT

In this experiment, *Aspergilus niger* is used to produce citric acid in surface fermentation process. In surface cultures technique, usually by batch wise process, fermentation broths have been prepared from, glucose syrup or molasses, wheat bran, potato starch in concentrations around 15% or high concentration of sucrose 15 -20% is used as carbon and energy source along to a substrate of citric acid. During the trophophase (first 30 hours), substrate glucose is mainly used for biomass production and completely oxidized to CO_2 via respiration, whereas during the idiophase losses by respiration are minimal and maximum substrate is converted to organic acids. The strain has accumulated high amount of citric acid after seven to fifteen days of incubation. The ratio of sugar consumed to amount of citric acid produced is normally observed in the ratio of 1:1. In static condition, fungi grow on the surface of the liquid media and developed thick mycelia mat, which releases the citric acid into the medium. At the beginning, the fermentation media is clear transparent and also in neutral pH, due to its production, colour of the fermentation media become pale yellow and pH turn to highly acidic. Quantification of citric acid is by acid base titration method or Furth-Herrmann Reaction (acetic anhydrous with pyridine).

10.3.1 Titration of Citric Acid

It is an acid-base titration method. Using acid-base indicator amount of acid available in the fermented media will be calculated. Spore un-inoculated culture media should be used as negative control to neutralize the initial acidity of the medium.

Citric acid is a polyprotic acid (can release three H^+s) that is a bit on the weak side (i.e., tends not to ionize completely). In solution in fruit juices, it lets a small portion of the H^+ go, however this small amount of acid is enough to create a pH = ~3 solution and a sharp taste on the palate. If strong base is added to citric acid it will sequentially lose its three protons. In this experiment use a solution of NaOH to titrate the acid in the fermented medium. Phenolphthalein is the indicator used in this procedure.

NOTES

10.3.2 Spectrophotometric estimation of citric acid

Furth-Herrmann Reaction: The Furth-Herrmann reaction consists simply in taking up the deproteinized sample in warm acetic anhydride and adding pyridine. Citric acid gives a carmine-red color, aconitic acid violet'-red, and tartaric acid emerald-green. The reaction must be carried out under anhydrous conditions.

10.4 MATERIALS REQUIRED

1. Spore suspension of *Aspergillus niger*
2. Citric acid production media
3. Conical Flask
4. Inoculation loop
5. Pipettes
6. Incubator
7. Centrifuge
8. Test tubes
9. Acetic anhydrous
10. Pyridine (anhydrous)
11. Spectrophotometer, etc.

10.5 PROCEDURE

10.5.1 Preparation of *Aspergillus niger* spore suspension

1. Prepare sterile potato dextrose agar plate.
2. Single spot inoculation of a spore of a pure culture of *Aspergillus niger* at the centre of the plate.
3. Incubate the plates for 48 hours.
4. Collect the black spores at the well developed Aspergillus colony and dissolve it in sterile distilled water.
5. Mix thoroughly and centrifuge at 6,000 rpm for 10 minutes.
6. Collect the spore pellet and re-dissolved in sterile Distilled water to make ~10^8 spores/ml [Spore suspension].

10.5.3 Citric acid fermentation

1. Prepare citric acid production media 100ml in 250 ml Erlenmeyer flask.
2. Sterilize the media by autoclaving 120^{o}C for 20 min.
3. Cool the production media. When it reaches the room temperature 2.o ml of the spore suspension of the Aspergillus niger is add aseptically.

NOTES

4. Mix thoroughly and incubate the culture media under shaking condition for 30hours.
5. After 30 hours of incubation, incubate the culture media at 25-28°C in a biological incubator for 7 to 15 days under static condition. Don't disrupt the culture during the incubation period.
6. After 7 to 15 days of incubation observe the colour change and pH change of the media using visual observation and also by using pH-meter.
7. Centrifuge the media at 8000 rpm for 20 minutes [removal of mycelia biomass].
8. Collect the supernatant for quantification of citric acid content by the following method.

10.5.4 Assay the citric acid content - Titration of Citric Acid

1. Prepare 0.1N NaOH as burette solution.
2. Take a clean dry 25 ml burette. Fill with standard 0.1N NaOH solution.
3. Take 10 ml of fermented media supernatant in a 100 ml conical flask.
4. Add a few drops of phenolphthalein indicator. Mix thoroughly.
5. Titrate against the NaOH solution, drop by drop addition.
6. Find the colour change end point. Repeat the titration process to get constant reading (A).
7. Similarly, un-inoculated sterile media also titrate against the standard NaOH solution (B)
8. Calculate the concentration of citric acid produce.

10.5.5 Spectrophotometric estimation of citric acid

1. Prepare standard graph of citric acid:
2. Prepare a citric acid stock solution at the concentration of 100mg in 100ml (mg/ml; 1000 µg/ ml).
3. Prepare working concentration of citric acid at 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µg/ ml.
4. Transfer 1 ml of the working concentration of the citric acid solution into a new clean dry test tube and add 8 ml of anhydrous reagent of acetic acid anhydrous and close the tubes with marbles/ cap.
5. Place the tubes in 60°C water bath for 10 minutes.

6. Add one ml of reagent grade pyridine in each tube and immediately close the tubes, and again incubated at 60^{o}C water bath for 40 min.
7. Transfer the tubes to ice-bath for 5 minutes (cooling).
8. Take spectrophotometric reading at 420nm, against the blank without citric acid.
9. Plot the values and draw the standard graph.
10. Perform the similar reaction to the test sample as the culture supernatant, and calculate the concentration of citric acid.

10.6 OBSERVATION & RESULT

10.6.1 Citric acid estimation by titration method

S. No	Volume of NaOH	Volume of acid Solution	Concentration

Calculations

Calculate the moles of citric acid present in the fermented media are as follows;

M1 V1 = M2 V2

M1 = M2 x V2/ V1

M1 = concentration of acid; V1 = volume of acid

M2 = Concentration of base; V2 = Volume of base

V2 = [A-B]

Normality of Citric acid = normality of NaOH× NaOH volume ÷ volume of Citric acid

• Concentration of Citric acid = Citric acid normality ×equivalent ×100 ÷ volume of distillation

• (Equivalent = 96, volume of distillation = 10)

10.6.2 Quantification of Citric acid by spectrophotometry

Si.No	Concentration of citric acid (µg/ml)	OD at 420nm
1	100	
2	200	
3	300	
4	400	
5	500	

6	600	
7	700	
8	800	
9	900	
10	1000	
11	Test-1	
12	Test-1	

NOTES

Plot the values in a graph. X- axis citric acid percentage and Y- axis OD value.

Result: Amount of citric acid present in the fermented media is ---------.

Interpretation

10.7 LET US SUM UP

In this experiment, you have learnt about the principles and physiology of citric acid fermentation using *Aspergillus niger*. The principle of acid titration and spectrophotometric quantification of the citric acid also learnt through this experiment.

Note: **Citric acid recovery:** The first step of citric acid recovery involves the precipitation of oxalic acid, possibly in the form of calcium oxalate at low pH, and subsequent separation from the medium containing the mycelium through rotating filters or centrifuges. Citric acid is then precipitated at pH 7.2 and 70-90 °C and recovered by filtration and drying. If a purer product are desired, it is dissolved with sulfuric acid, treated with charcoal or ion exchange resins, and again crystallized as anhydrous citric acid (above 40 °C) or as a monohydrate (below 36.5 °C). In surface processes, the mycelium is sometimes squeezed to increase the recovery yield.

10.8 UNIT - END EXERCISES

1. How citric acid is fermented?

2. How titration method used for citric acid estimation?

3. Demonstrate the protocol for Furth-Herrmann Reaction.

10.9 SUGGESTED READING

1. Dinithi Dahanayake. Estimation of citric acid from *Aspergillus* sp. document/99107113/2012**.**

2. Bio-Resource: Technical Resources in Biotechnology. 2012. Citric Acid Production by *Aspergillus niger*, Estimation of Citric acid. technologyinscience.blogspot.com

3. CRC Handbook (1973-74): Concentrative properties of aqueous solutions - sodium hydroxide.

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UNIT – 11: SEPARATION OF BIOMASS – WET AND DRY BIOMASS PREPARATION

Structure

11.1 INTRODUCTION

Microbial biomass is a fundamental variable in industrial microbiology. While growth for muticelluar organisms is typically measured in terms of the increase in size of a single organism, microbial growth is measured by the increase in population, either by measuring the increase in cell number or the increase in overall mass. Four types of microorganisms are used to produce biomass: bacteria, yeasts, fungi and algae for various industrial bioprocesses. Product fermentation is directly related to the growth of microorganism used. To recover the fermented metabolite and estimation, harvesting the total biomass is important one for determination of the microbial productivity (g of metabolite / g of biomass).

Biomass is the microbial cell population commonly expressed as dry weight, is a parameter that may be required for the determination of growth kinetics. The traditional method used for the determination involves an oven-drying step and equilibration to room temperature before weighing. It is tedious and time consuming. Besides, the choice of a biomass analysis of microorganism depends on numerous criteria, most importantly based on the type of culture, nutritional requirements, and the selection of separation technique.

For the biomass analysis, cells should be separated by different techniques from the fermentation broth. Conventional biomass

estimation techniques are indirect, typically relating physical (optical density, turbidity, capacitance) or metabolic (oxygen uptake, CO_2 evolution) values to the total biomass expressed as wet weight or dry weight. The traditional standard method for the direct determination of biomass by dry weight measurement includes drying a sample to constant weight in a conventional oven. This method generally requires lengthy drying phases as well as an equilibration step where the sample temperature is reduced to room temperature in a desiccator. Accordingly, results are not rapidly available to allow prompt utilisation of biomass-based information. The dry biomass of the culture can be calculated based on the percentage of Dry Biomass, divide it by 100 and then multiply by the quantity of Wet Biomass that you have on a surface.

For the biomass determination, the biomass of the culture media should be harvested. Clarification and sedimentation are the methods used for harvesting suspended microbial biomass in the fermented medium. Clarification is a method of removing suspended particles and separating clear liquids from cloudy solids/ biomass. Centrifugation is the effective method used to sediment out the suspended microbial cells/mats. Centrifugation is a technique used for the separation of particles from a solution according to their size, shape, density, viscosity of the medium and rotor speed. The particles/cells are suspended in a liquid medium and placed in a centrifuge tube. The tube is then placed in a rotor and spun at a define speed. Separation through sedimentation could be done naturally with the gravitational force. Centrifugation is making that natural process much faster. Rotation of the rotor about a central axis generates a centrifugal force upon the particles in the suspension. The rate of centrifugation is specified by the angular velocity usually expressed as revolutions per minute (RPM), or acceleration expressed as g. The particles' settling velocity in centrifugation is a function of their size and shape, centrifugal acceleration, the volume fraction of solids present, the density difference between the particle and the liquid, and the viscosity. Microbial cells can be sedimented by this method and the supenatent is discarded, dry in oven at ambient temperature, after drying the cells will be estimated at room temperature using weighing balance. In this method, cultured yeast cells wet and dry biomass will be calculated.

NOTES

11. 2 OBJECTIVES

After going through this Experiment you will be able to understand;

- The wet weight of the microbial biomass.
- The moisture content of the microbial biomass.
- The dry biomass of the cultured microorganism.

11. 3 ABOUT THIS EXPERIMENT

In this experiment, wet weight and dry weight of the bacterial biomass can be calculated. The basic tool like centrifugation is used, by which the suspended bacterial biomass can be deposited and calculated the wet biomass, dry in ho air oven to remove the moisture content and calculate the dry weight.

11.4 MATERIALS REQUIRED

1. Microorganism: Yeast cells
2. YPD medium
3. Incubated shaker, Centrifuge, centrifuge tube, pipette, desiccators, weighing balance, etc.

11.5 PROCEDURE

1. Prepare 100 ml of YPD broth in 250 ml Erlenmeyer flask. Sterilize the medium at 120^{o}C for 20 min in autoclave.
2. One ml of log phase yeast culture in YPD broth (24hours culture) is inoculated in to the sterilized YPD broth reached at room temperature.
3. Incubate the broth at 35^{o}C for 48 hours in shaking incubator.
4. After incubation, take 10 ml of the well mixed yeast culture in to a sterile 15 ml pre- weighted centrifuge tubes (**A**).
5. Centrifuge the culture tubes at 8000 RPM for 15 minutes.
6. Gently remove the supernatant from the centrifuge tubes.
7. Add 5 ml of sterile saline and resuspend the cell mass, and follow the centrifugation process again.
8. Decant the supernatant, and gently dry the cell mass (excess water) for 2.0 min at room temperature.
9. Take the wet weight of the biomass along with the centrifuge tube (**B**).
10. Place the centrifuge tubes in hot air oven at 45 to 60^{o}C for 4 to 8 hours to complete drying.

11. After drying, the centrifuge tubes are kept in desiccators for the tubes reach the room temperature.
12. Weigh the dry mass of the cell pellet along with the tube (**C**).
13. Calculate the wet and dry biomass of the culture by the following methods.

11.6 OBSERVATION & RESULT

Biomass determination

Tube No.	Pre-weight (g) [A]	Wet weight (g)		Dry weight (g)		Moisture content [D-E]
		Along with tube [B]	[D] = [A-B]	Along with tube [C]	[E] = [A-C]	

Calculation:

Wet weight of the cell biomass = B – A/volume of culture used = g wet biomass/ml of culture (D)

Dry biomass of the cell biomass = C - A/ volume of culture used = g dry biomass/ml of culture (E)

Moisture content (%) of the Biomass = (D) – (E)/ (D) * 100 = -----% of moisture.

Dry mass percentage = (dry biomass weight (g) – wet biomass weight (g) x 100

Result:

1. Wet weight of the given culture biomass = ________ g/ml.
2. Dry weight of the given culture biomass = ________ g/ml.
3. Moisture % = ______ .

Interpretation

____

____

____

____

11.7 LET US SUM UP

In this experiment, you have learnt about the biomass determination of the bacteria from the fermented medium. Biomass determination is used for calculation of the industrial strains fermenting ability like, productivity/biomass ratio.

10.8 UNIT - END EXERCISES

1. What is biomass? How do you determine the biomass?
2. Differentiate wet biomass and dry biomass?
3. Explain the concept of centrifugation.

11.9 SUGGESTED READING

1. Bernhard Sonnleitner, Georg Locher, Armin Fiechter.Biomass determination-Minireview. Journal of Biotechnology, 25 (1-2), 1992: 5-22.
2. E. Li, R. Mira de Orduña. A rapid method for the determination of microbial biomass by dry weight using a moisture analyser with an infrared heating source and an analytical balance. letters in Appl. Microbiol. 2010. https://doi.org/10.1111/j.1472-765X.2009.02789.x

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UNIT – 12: IMMOBILIZATION TECHNIQUE – CELLS AND ENZYMES

Structure

12.1 INTRODUCTION

12.1.1 Whole Cell Immobilization with Calcium Alginate

Immobilization defined as making something immobile or fixed. It defined that immobilized biocatalysts, (or) enzymes, (or) cells physically fixed in a defined region in a solid support/ matrix in order to catalyze a specific reaction with no loss of activity and with repeated use. Naturally, cells like prokaryotes are move to access the free nutrients for its growth in its microenvironment. Most microorganisms are likely to attach themselves onto a solid surface (biofilm) for its growth and survival. For our purpose, either whole cell or the enzymes are immobilized in a suitable matrix, which may have a higher biocatalytic property, long term stability, low or no loss of activity and regenerating possibility. The matrix is usually a high molecular weight polymer such as polyacrylamide, starch, cellulose, etc. The advantage of immobilizing enzymes or cells over free cells is to increase their stability, efficiency and also have low level of cell leakage and high resistance to abrasion. The immobilized enzymes or cells can also be recovered at the end of the reaction and can be used repeatedly.

Immobilization Techniques

In 1916, Nelson and Griffin discovered the invertase enzyme that shows the same activity when absorbed on a solid surface as when uniformly distributed throughout the solution. This was the first evidence of enzyme immobilization technique. An enzyme is usually immobilized onto an inert, insoluble material e.g. Calcium alginate. This is produced by the reaction of a mixture of Sodium alginate solution with Calcium chloride. These beads provide increased resistance to changes in conditions such as pH or temperature. They also allow enzymes to be available in place throughout a reaction, following which they are easily separated from the products and may be used repeatedly.

Immobilization techniques improve enzyme properties by increasing stabilization and rigidity of 3D structure, causing chemical modification, generating hyper-hydrophilic microenvironments and reducing enzyme inhibitions. There are four principal methods available for immobilising enzymes/cells:

a. adsorption
b. covalent binding
c. entrapment
d. membrane confinement

Physical adsorption: Physical adsorption is the one of the simple and less expensive easiest techniques which retains high catalytic activity, as a result, commonly used immobilization in industry. In physical adsorption the interaction may be occurs by hydrophobic interactions, van der Waals forces, or hydrogen bonding and dipole-dipole interactions are used for immobilization. The process is depends on the natural properties of the substrate surface and the adsorbate. This technique also allows the reuse of expensive support materials, this reusability of the support material brings economic advantages for industrial productions. On the other hand, this technique does not offer high stability and might cause loss of biomolecules that is immobilized during washing and operation.

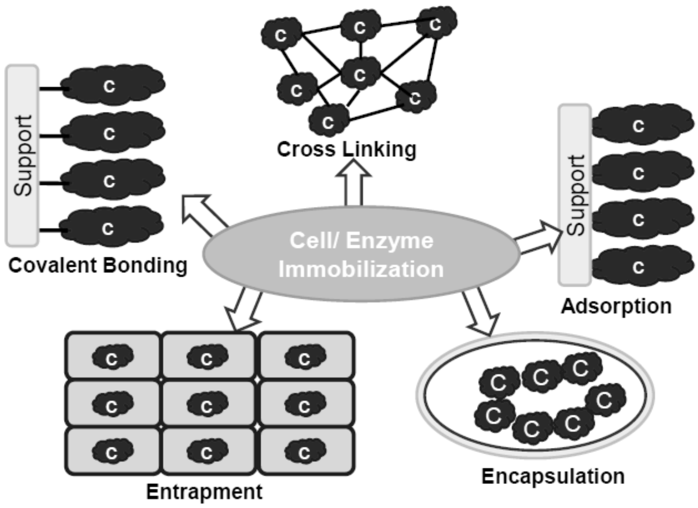


Figure -12.1: Methods of immobilization technique.

Encapsulation or entrapment: The encapsulation process is based on the entrapment of the biomolecule in a high molecular polymeric matrix. The main advantage of this technique is that it allows the exchange process of low molecular weight compounds through the permeable matrix. Entrapment matrix is generally formed during the immobilization process. Therefore, the properties of the gel matrix and the conditions used for the encapsulation should be compatible with the enzyme/ cells to be immobilized.

Cross-linking method: Cross-linking is an immobilization technique that combines both covalent bonding and entrapment. Immobilization by this method requires crosslinking agents (polymers) such as glutaraldehyde and bisisodiacetamide. The enzyme activity of immobilized preparations cross-linked with these reagents is dependent upon the degree of cross-linking and the active site exposure (enzymes).

Covalent binding: Covalent binding is based on the formation of a covalent bond between the enzyme catalyst / cells and the support material. Covalent binding causes a tight binding (strong) so the catalyst to be immobilized does not separate from supports during utilization. Due to its stronger interaction, it is highly heat stable. However, this interaction, unfortunately, does not allow the enzyme molecules to have free movement, resulting in decreased enzyme activity. This technique is less effective for immobilization of cells, and support materials are not renewable.

12.1.2 Enzyme immobilization

Immobilized enzymes have lot of commercial applications which includes, 1) convenience to use, upon completion, reaction mixtures typically contain only solvent and reaction products; 2) economic process, because the immobilized enzyme is easily removed from the reaction making it easy to recycle the biocatalyst; and 3) more stable in thermal and physical properties than free soluble enzymes. In addition, the immobilization approach together with enzymes has been the enzymatic reactions on immobilized substrates. This approach facilitates the analysis of enzyme activities and mimics the performance of enzymes on e.g. cell walls.

Various methods of immobilization have been developed, including adsorption, entrapment, encapsulation, covalent binding and cross-linking. Selection of most suitable immobilization method is important, it directly influence the structure and function of an immobilized enzyme. The supportive material used in the immobilization method is generally divided into organic, inorganic and hybrid or composite. The support material should protect the enzyme structure against adverse reaction conditions which help the immobilized enzyme to retain its catalytic activity. However, it has some limitations, due to the negative effect of matrix on the structure of the enzyme. Hence, suitable supportive materials are required to retain enzyme activity. The matrix must have the following desirable properties such as, 1) biocombatiable, 2) high affinity to enzymes, 3) Presence of relative functional groups, 4) regeneration and reuse, 5) thermal stability, and 6) Insoluble during reaction. There are two type of matrix materials are used, such as 1) inorganic and 2) organic.

In inorganic matrix used for immobilization of enzymes include;

1) Silica and Inorganic Oxides: Presence of many hydroxyl groups on the surface of silica facilitates enzyme attachment and favours its functionalization with surface modifying agents. Modified form of glutaraldehyde or 3-aminopropyltriethoxysilane (APTES) is used for enzyme immobilization.

2) Mineral Materials: Presence of many functional groups (such as –OH, COOH, C=O, –SH, $–NH_2$) on the surface of the minerals allows the formation even of covalent bonds between the enzyme and the support and facilitates modification of the minerals.

3) Carbon-Based Materials: Activated carbons and unmodified and modified charcoals have been used as effective and valuable support materials in enzyme immobilization, due to high adsorption capacity, the abundance of many functional groups and minimal release of fine particulate matter make carbon-based materials suitable carriers for the adsorption immobilization of various enzymes.

Organic materials are broadly in to two types they are,

1) Synthetic Polymers: A very wide range of verified chemical functional groups may be available to make interaction with enzymes. For example, carbonyl, carboxyl, hydroxyl, epoxy, amine and diol groups. In addition, strongly hydrophobic alkyl groups and trialkyl ammine moieties also available in this polymers. The type and quantity of functional groups determine the hydrophobic/hydrophilic character of the matrix and therefore its ability to form polar or hydrophobic interactions with the enzyme. E.g Amberlite and Sepabeads— alcohol dehydrogenase; glutaraldehyde – laccase; polyurethane foam – inulinase; polyamide 66 - *a*-amylase.

2) Biopolymers: Carbohydrates and some proteins such as albumin and gelatin. Materials like collagen, cellulose, keratins and carrageenan as well as chitin, chitosan and alginate are examples of biopolymers. It contains hydroxyl but also an amine and carbonyl moiety—enables direct reaction between the enzyme and matrix and facilitates modification of their surface. Chitosan is also used for various enzyme immobilizations.

In this experiment, whole cell immobilization using yeast cells in a calcium alginate beads for invertase activity.

12. 1.2 OBJECTIVES

After going through this Experiment you will be able to understand;

- The formation of immobilized yeast cells using alginate polymer.
- The use of immobilized cells.
- Agar or agarose, calcium chloride dehydrate
- α-amylase (fungal / bacterial origin)
- Pasteur pipette, aseptic chamber, Glassware, etc.
- Soluble starch, and 3,5-dinitrosalicylic acid (DNS), Double-distilled water, phosphate buffer.

NOTES

12.3 ABOUT THIS EXPERIMENT

12.3.1 Whole Cell Immobilization

In this experiment, whole cell immobilization using yeast cells in a calcium alginate beads for invertase activity will be performed. The activity of the immobilized cells will be determined by the increase of reducing sugars in the fermented media.

The basic principle of the reducing sugar estimation is given in the chapter- 5, section 5.2.2.

12.3.2 Enzyme immobilization.

In this experiment, immobilization of α-Amylase enzymes by using agarose gel matrix. Generally, a wide variety of supports have been used for immobilization of amylases. Most of which modify the enzyme chemically, hindering the enzyme functions. Physical entrapment of α-amylase in cross-linked biodegradable hydrogels like calcium alginate and kappa-carrageenan beads has been shown to be a relatively rapid, easy, and safe technique. Normally, calcium alginate and kappa-carrageenan get ionized in acidic or slightly acidic environment. Thus, beads lose their structural integrity and functional reusability of the enzyme. But, agar is resistant to acidic hydrolysis. Agar is a naturally occurring heterogeneous colloidal polysaccharide complex of agarose and agaropectin having alternating α-(1→3) and β-(1→4) linkages. The structure of agaropectin is not fully known but it is a sulphonated polysaccharide in which galactose as well as uronic acid is partly esterified with sulphuric acid. However, agar has the ability to form gel at salt free condition. It is the polymer of choice for entrapment of cells and enzymes because of its cost effectiveness and resistance to acidic hydrolysis. Agar also offers a relatively inert aqueous environment within matrix. Based on the above basic information's, in this experiment, to entrap α-amylase in agarose gel and stabilize in calcium chloride solution.

12.4 MATERIALS REQUIRED

1. Yeast potato Dextrose (YPD)/ Potato Dextrose Agar (PDA) medium (for cultivation of Yeast cells)
2. Sodium alginate solution (2.5% w/v in 0.1% NaCl)
3. Gluteraldehyde (2.5% v/v)
4. Calcium Chloride ($CaCl_2$) solution (0.05 N)
5. Sucrose solution (1% w/v)

6. DNS reagent, phosphate buffer (pH 7.0)
7. Spectrophotometer, beaker, Pasteur pipette, conical flask, glass column, etc.
8. Agarose solution (2 - 4 %)

12.5 PROCEDURE

12.5.1 Immobilization of Yeast Cells

1. Prepare 100 ml of YPD medium in 250 ml flask and autoclaved at 120^{o}C for 20 min.
2. Scrap the yeast cell biomass (single pure colony) from the well grown cells of yeast on the pure culture plate/ slant of PDA medium, inoculate in YPD medium.
3. Incubate the culture flask in a room temperature (30^{o}C) for 24 hours under shaking condition (100 rpm).
4. Take 20 ml of the yeast culture in 30 ml centrifuge tubes (sterile) and centrifuge the cells at 8000 RPM for 15 minutes.
5. Remove the supernatant and resuspend the cells using 0.1% sterile NaCl solution (wash).
6. Centrifuge the cells at 8000 RPM for 15 minutes, repeat in thrice [get salt &contaminant free yeast cells). [Optional]
7. Final cells should be making up to 1.0ml using sterile 0.1% NaCl.
8. Prepare 10 ml of sodium alginate solution and add 1 ml yeast cell in it.
9. Mix properly and incubate at room temperature for 30 minutes then add 1.0 ml of gluteraldehyde solution incubate at room temperature for 90 minutes.
10. With the help of 10 ml pipette, drop wise add this mixture into the beaker containing 100 ml $CaCl_2$ solution.
11. Filter out the beads with the help of normal filter paper.
12. Wash the beads 2-3 times with sterile Distilled water.
13. Load these beads into thoroughly washed glass column/ beaker.
14. Add 50ml of sterile 1% sucrose solution.
15. After every 30 minutes interval collect the 1 ml of sample and estimate the amount of glucose by using Dinitrosalilcylic acid method (DNSA) method

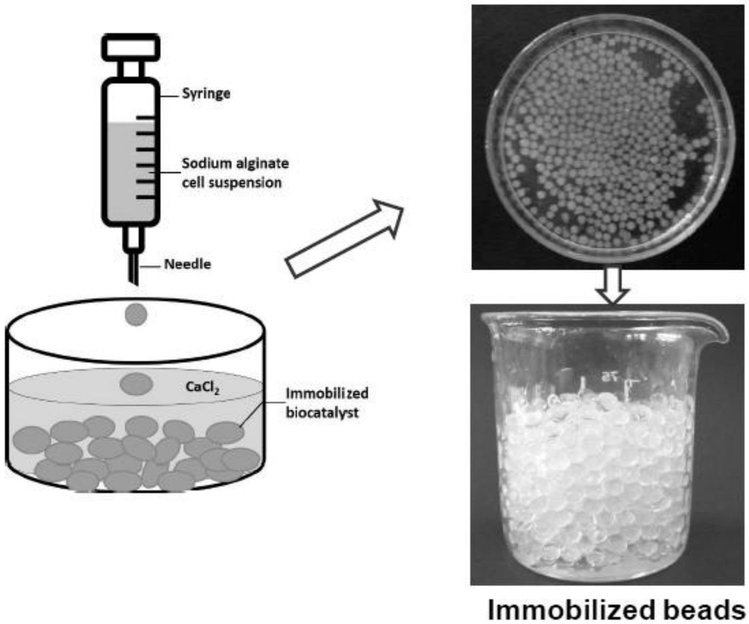


Figure-12.2: Calcium alginate based immobilization technique.

12.5.2 Glucose estimation by Dinitrosalilcylic acid method (DNSA) method

1. Prepare a standard graph of glucose solution [0.1 to 1.0 mg/ml]- Refer Experiment No. 5.3.1
2. Prepare water blank.
3. Take three tubes and labels as T1, T2 & T3 & pipette out 3 same volume (0.5ml) of sucrose solution from reaction mixture from above experiment in this tube
4. Add distilled water in all tubes in such a way that the total volume will be 2.0 ml
5. Add 2.0 ml of DNSA reagent in all tubes. Mix it properly by reversing the tubes or by using magnetic stirrer
6. Keep all the tubes in boiling water bath for 10 minutes. Then allow it to cool down
7. Take absorbance at 540 nm (using green filter) and plot a standard curve
8. Calculate out the concentration of glucose produced in the reaction mixture of above experiment

12.5.3 Enzyme Immobilization

1) Prepare agar or agarose solution (2–4% w/v): Take suitable concentration of agarose (2-4%) in clean chemical free conical flask

and heating the agar powder dispersion in phosphate buffer (pH 6.8) at 90°C.

2) Cool the melted agarose in to 45°C.
3) Add required quantity (equal volume) of enzyme (1.0 mg/ml of α-amylase) solution in agar solution. Mix the content thoroughly [care should be taken at the time of mixing to avoid gas bubbles).
4) Take the enzyme mixed agar in to a 20 G × 1.5 in sterile syringe.
5) Release the agar mixture into a sterile calcium chloride (100 mM) solution drop by drop (Fig.12.3).
6) Allow the beads to harden for 10–30 min.
7) Wash the beads in sterile distilled water minimum of two times.
8) Dry to get constant weight in a desiccator at room temperature for 48 h.

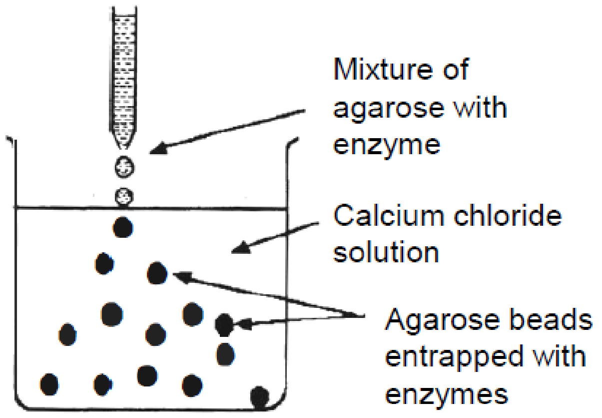


Figure-12.3: Enzyme immobilization in entrapment method.

12.5.4 Immobilization efficiency test

1) Estimate the total protein at the initial time of use.
2) Collect the filtered distilled water (bead wash water) and the calcium chloride solution.
3) Estimate the total protein content of the wash & calcium chloride solution (Follow Determination of Protein Content by Bradford's method).
4) Calculate the immobilization efficacy by the following formula:

Immobilization efficiency (%) = [(Theoretical enzyme loaded − Amount of enzyme leached in calcium chloride solution and washings)/Theoretical enzyme loading] × 100.

12.5.6 Estimation of Protein Content by Bradford's method

1) Prepare the protein standard graph using 10 to 100 µg/mL of protein (BSA).
2) Take a series of 12 test tubes. Each tube with 1ml of different concentration of protein solution and add 1ml of 0.1 M phosphate buffer pH 7.5.
3) Add 5 mL Bradford's reagent (0.1 g of coomassie brilliant blue G 250 dissolved in 50 mL of ethanol + 100 mL 85% phosphoric acid and volume was made to 1 L).
4) Wait for 2 minutes to develop colour completely.
5) Take the absorbance at 595 nm against blank (without protein).
6) Plot the standard graph.
7) Similarly, take 1ml of enzyme (initial) and the wash & calcium chloride solution. Add 1ml of phosphate buffer pH 7.5.
8) Follow the steps 3 to 5, and calculate the total protein at before and after immobilization.

12.5.7 Amylase Enzyme Assay

Refer the protocol of the Unit-7, section 7.4

12.6 OBSERVATION & RESULT

Sugar Standard Graph & Immobilized cell activity determination

Si. No	**Concentration of Glucose (mg/ml)**	**OD at 540 nm**	**Si. No**	**Concentration of Glucose (mg/ml)**	**OD at 540 nm**
1	0.1		B	--	
2	0.2		T1- 30 min		
3	0.3		T2 - 30 min		
4	0.4		T3 - 30 min		
5	0.5		T1 - 60 min		
6	0.6		T2 - 60 min		
7	0.7		T3 - 60 min		
8	0.8		T1 - 90 min		
9	0.9		T2 - 90 min		
10	1.0		T3 - 90 min		

B – Blank; T1 – T3 – Test samples.

NOTES

Result

Increase in the concentration of glucose from the sucrose with respect to time by Active yeast cell (Invertase) indicates that Yeast Cells immobilized and they are viable.

A) Number of beads developed/ ml of the agar solution =

B) Immobilized Enzymes: Protein concentration

Si no.	Protein concentration (µg/ml)	D value at 595 nm	Si no.	Protein concentration (µg/ml)	OD value at 595 nm
1	10		8	80	
2	20		9	90	
3	30		10	100	
4	40		**Initial**		
5	50		**In Wash**		
6	60		**In $CaCl_2$**		
7	70				

C) Immobilized Enzymes: Amylase activity – DNS method.

Si no.	Glucose concentration (µg/ml)	D value at 595 nm	Si no.	Glucose concentration (µg/ml)	OD value at 595 nm
1	100		8	800	
2	200		9	900	
3	300		10	1000	
4	400		**Initial**		
5	500		**30 min**		
6	600		**60 min**		
7	700				

RESULT

Initial activity of the free enzyme = ------- U/ml/min
Volume of enzyme solution = ----- ml
Weight of beads formed after immobilization of enzyme solution = ------- g
Enzyme solution entrapped in 0.5 g beads = -------- ml
Therefore enzyme entrapped = ------- U/0.5g beads (on the basis of free enzyme)
Activity of immobilized enzyme obtained in 0.5 g beads = ------- U/min

NOTES

Therefore Total Enzyme activity after entrapment = -------%
Total amylase activity /bead = total amylase activity U/ml / number of beads = ------ U/bead

Interpretation

____
____
____
____

12.7 LET US SUM UP

In this experiment, you have learnt about the immobilization techniques. Both whole cell as well as enzyme immobilization has been studied. Viability of the immobilized cells and the enzymes also studied.

12.8 UNIT - END EXERCISES

1. What is immobilization? Explain the types of immobilization?
2. What is a whole cell immobilization? Explain.
3. How enzymes are immobilized?
4. Demonstrate the functionality of immobilized enzymes.

12.9 SUGGESTED READING

1. Microbiology- A laboratory Manual-International student Edition: 4th edition. By James G. Cappuccino& Natalie Sherman
2. Bacteriological Techniques By F.J. Baker
3. Introduction to Microbial Techniques By Gunasekaran
4. Manual of Industrial Microbiology and Biotechnology, 2nd Edition by Arnold; . Demain and Julian, E. Davie Ronald M. Atlas, Gerald Cohen, Charles L. Hershberger, Wei-Shou Hu, David H. Sherman, Richard C. Wilson and J. H. David Wu.

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PART – 2

MICROBIAL BIOTECHNOLOGY

UNIT – 13: ISOLATION OF DYE DEGRADING MICROORGANISM

Structure

13.1 INTRODUCTION

Environmental pollution has been recognized as one of the major hazard of the recent world. Ever increasing human population, the demand for human need is increased. To meet out the urgent requirements, large number of food, textile and pharmacy industries have been emerging, where lot of chemicals including dyes were manufactured and used in day to day life. Dyes are usually a chemical compounds with a complex synthetic origin and are more stable in the environmental conditions. Worldwide over 0.7 million tons of 10,000 different synthetic dyes and pigments are used industrially. Due to urbanization and development the use of dyes and dye based products grows, and larger amount of these dyes are released to the environment as spent/ effluent. Once they enter an ecosystem, it become persist and initiate a series of environmental issues both its biotic and abiotic elements.

Dyes are composed of a group of atoms known as chromophores, responsible for the dye colour, ad are classified according to their chemical structure and applications. Azo, anthraquinone and phthalocyanine dyes are the three most common groups of dyes, which are toxic and harmful for the environment. Disposal of these dyes causes serious environmental damage, including

inhibition of aquatic life forms and terrestrial fauna and flora. One of the serious causes of environment is due to dye effluents to the water bodies and the agricultural fields. Dyes in aquatic habitat reduce the light penetration, dissolved oxygen, and an increase of toxicity to fauna. Contamination of soil with dyes deteriorates change its physicochemical properties and decreases fertility of soil.

Textile dyes like azo dyes are usually treated by physical or chemical treatment process which includes physical or chemical flocculation, electroflotation, electro kinetic–coagulation, membrane-filtration, electro chemical-destruction, flotation and precipitation. These methods are resourceful but, it secondary waste like pollutants and they are inefficient and expensive. Hence, an alternative and efficient method with less toxicity is a requirement today. Bioremediation is an economically viable safe and slower process carried out by both aerobic and anaerobic microorganisms. Variety of microorganisms in nature has potentials to utilize and degrade the toxic dyes as their carbon and nitrogen requirements. Microbes produce several enzymes like azo reductase, peroxidases, laccase and other peroxidase like enzymes by which they have utilize and either degrades or transforms the toxic substances and become nontoxic end products. Some time, microbes completely oxidize the waste pollutants for its energy requirements.

Bacterial genera, such as *Pseudomonas*, *Bacillus sp.*, *Rhodococcus*, *Kocuria rosea*, *Rhizobium radiobacter*, *Exiguobacterium* sp., *Proteus* sp. are reported for rapid azo dye decolorization. The above said organisms are the producer of oxidoreductive enzymes, such as lignin peroxidase, laccase, tyrosinase, MG reductase, azoreductases and other non specific reductases. In anaerobic degradation, the enzymes of microorganisms involve reductive reaction on azo bonds by the transfer of reducing equivalents resulting in the formation of aromatic amines. Facultative microorganisms largely carried out such reductive amine formation process.

The bacterial degradation of azo dyes in either aerobic or anaerobic conditions have been associated with symmetric cleavage of the azo group (–N=N). The process of this cleavage is mediated by different mechanisms, such as enzymes, chemical reduction, low molecular weight redox mediators, or a combination of both. The reductive reaction could be either intracellular or extracellular.

Anaerobic azo reductases are flavoprotiens (NAD(P)H:flavin oxidoreductase), localized at intracellular or extracellular site of cell membrane. It requires NADH, NADPH and FADH as electron donors (co-factors) for reduction of azo bonds, which provide 'H' for reduction. For example, figure-1 shows the methyl red is catalyzed by the azo reductases to produce 2-Amino benzoic acid and p-dimethyl amino aniline. The degraded product is a colourless end product, so the colour of the dye becomes colourless.

13. 2 OBJECTIVES

After going through this Experiment you will be able to understand;

- The dye degrading microorganisms from the soil sample.
- The efficiency of dye degradation [broth culture method].

13. 3 ABOUT THIS EXPERIMENT

In this practical, the potential azo dye degrading bacteria can be isolated from the dye contaminated soil sample. Using simple serial dilution method followed with spread plate / pour plate technique the dye degrading bacteria can be isolated. For this, isolation media is prepare with a supplement of (0.01 to 0.1%; w/v) azo dye, following bacterial inoculation and incubation, bacteria grow on dye containing media and produce colourless clear zone around it, which indicates positive sign for dye degradation. The larger zone of dye clearance isolate is selected for further conformation and identification.

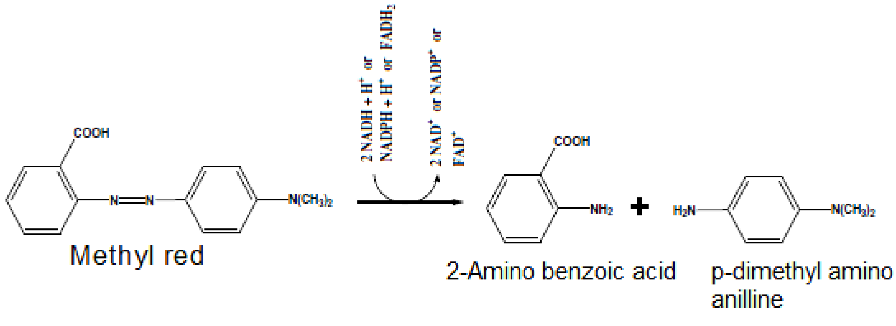


Figure -13.1: Methyl red is degraded by azo reductases enzyme.

Further, variable concentration of azo dye containing medium is inoculated with azo dye decolourizing bacteria. During the bacterial growth at static condition, the colour change of the medium will be

observed. The colour change is due to the reduction process of azo bonds on azo dyes by the bacterial azo reductase enzyme. The percent (%) colour change will be equivalent to the amount of enzyme produced by the bacteria. In this study methyl red is used for quantitative determination of dye decolourization (Fig.13.1)

13.4 MATERIALS REQUIRED

1. Nutrient media, Luria Bertaini media, minimal salt media.
2. Soil sample – sample collected from dye industrial waste.
3. Dyes for screening: Malachite green, Cango red,
4. Sterile saline water blanks (8 to 10 nos)
5. Incubator, inoculation chamber, Sprit lamp, L-rod, pipettes, etc
6. Dye degrading bacterial isolates (log phase culture)
7. Minimal salt broth with different concentration of azo dyes (0%, 0.01%, 0.025%, 0.05%, 0.1%, 0.25% of Methyl red)
8. Inoculation loop, Sprit lamp, incubator, etc.

13. 5 PROCEDURE

13.5.1 Screening of Dye Decolourizing Bacteria

1. Prepare two different types of media plates.
   a) Prepare ten nutrient agar plates (sterile). [Five plates with 0.1 or 0.05% w/v of the dye material, prepare other five plates without dye].
   b) Prepare ten minimal media with specific dye at (0.1% or ` 0.05%; w/v)
   c) Mark the media plates and keep this in the inoculation chamber.
2. Sample preparation and serial dilution.
   a. Take one gram of soil in to a 10 ml of sterile water blank. Vortex the content and allow to settle down for the soil particles.
   b. Perform a serial dilution procedure to transfer one ml of the soil suspension to the second tube and mix thoroughly.
   c. Repeat the step up to 10-7 dilutions.
3. Plating the sample
   a. Take 0.1 ml of the diluted soil samples (10^{-4} to 10^{-7}) and inoculate on the sterile media with and without dye.
   b. Using L-rod to spread the sample at the entire surface of the media.

c. Incubate the plates at 32 to 37^{o}C for 48 hours.
d. Observe the growth of the bacteria after the incubation period.

4. Observe the results.
   a. Observe the clear transparent zone around the bacterial colony, which represent the utilization of dye from the media.
   b. Count the total number of colonies and the decolourizing colonies.
   c. Compare the colonies on media with dye and without dye and record the results.
   d. Select any five to ten decolourizing colonies and maintain as pure culture.

13.5.2 Quantitative Determination of Dye Decolourization

1. Prepare mineral salt solution broth with variable concentration of azo dyes (0%, 0.01%, 0.025%, 0.05%, 0.1%, 0.25%), sterilize at 120^{o}C for 15 minutes by autoclaving.
2. After cooling, inoculate the culture tubes by 0.01 ml of test bacterial inoculums. Each concentration maintains uninoculated tube as control.
3. Incubate the culture tubes at 35 ±2 ^{o}C for 48 hours in static condition.
4. After incubation, centrifuge the culture tubes at 8000rpm for 10 minute; carefully collect the culture supernatant for determination of decolourization.
5. Using the control tube, detect the absorption maxima (λ max) in UV-Vis Spectrophotometer.
6. Using the λ max nm, the 0% uninoculated culture tube blank, estimate the residual dye colour in the culture tubes.
7. Calculate percentage of decolourization from the following equation,

   Decolorization (%) = (A –B x100) / A

   A – Initial OD; B – Final OD

13.6 OBSERVATION & RESULT

A) Screening of Dye Degrading Bacteria

Si. No.	Dilution factor	Plate name	Number of colonies [CFU]		% of Dye degrading organisms
			With Dye	No Dye	
1	10^{-3}				
2	10^{-4}				
3	10^{-5}				
4	10^{-6}				
5	10^{-7}				

Calculation: % Dye degrading organisms = Number of CFU with clear zone x 100 /Total number of colonies

Interpretation of the result

Positive result: Following bacterial inoculation and incubation, bacterial colony developed with colourless clear zone.

Negative result: Either no growth or the bacterial colony without dye clearing zone.

B) Efficiency of dye decolourization

Azo dye Concentration (%)	Initial OD [A]	Final OD [B]			Decolorization (%)		
		Isolate -1	Isolate -2	Isolate -3	Isolate -1	Isolate -2	Isolate -3
Control							
0.01							
0.025							
0.05							
0.1							
0.25							

RESULT

Number of dye decolourizing bacteria from the sample: ____ CFU/mL.
Percent decolourizing bacteria: _____.
Efficiency of dye [0.1% Methyl red] decolourization: _____ %.

Interpretation

13.7 LET US SUM UP

In this experiment, you have learnt about the dye decolourization bacterial isolation and screening method, followed by the efficiency of dye decolourization. The efficiency of methyl red dye decolourization is also learnt through this experiment.

13.8 UNIT - END EXERCISES

1. Discus dye decolourizing bacteria?
2. How do you determine the dye degrading bacteria?
3. How do you evaluate the efficacy of dye decolourizing bacteria.
4. Design an experiment to isolate azo dye degrading bacteria.

13.9 SUGGESTED READING

1. Microbiology- A laboratory Manual-International student Edition: 4th edition. By James G. Cappuccino& Natalie Sherman
2. Introduction to Microbial Techniques By Gunasekaran
3. Manual of Industrial Microbiology and Biotechnology, 2nd Edition by Arnold; Demain and Julian, E. Davie Ronald M. Atlas, Gerald Cohen, Charles L. Hershberger, Wei-Shou Hu, David H. Sherman, Richard C. Wilson and J. H. David Wu.

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UNIT – 14: ANTIBACTERIAL SENSITIVITY TEST

Structure

14.1 INTRODUCTION

A milestone in the history of medicine was the discovery of penicillin by Alexander Fleming in 1928. More than thousands of antimicrobial compounds discovered and have been used for the treatment of various infectious diseases. However, the development of antibiotic resistance in bacteria or other pathogens to the existing antimicrobials has been a raising serious problem for the treatment of infectious diseases. For knowing the sensitivity or resistance of antimicrobials to the pathogens is the urgent requirement for the treatment of infectious diseases. Hence, the physicians request the microbiology lab to quickly check the causative agent (pathogen) of the patient against various concentrations of a given known antimicrobial to determine susceptibility or resistance to that drug. Most of the clinical laboratories use either disk diffusion or agar well diffusion assay for the determination of antimicrobial susceptibility. But the broth dilution method is the gold standard procedure to determine the accurate concentration of the drug to inhibits or kill the pathogens completely.

The disk diffusion assay method had developed at the early 1950s for determining susceptibility of bacteria to antimicrobials. Each laboratory modified the procedure to costume its own requirements, which included the use of different media, varying inoculums concentration, differentiate incubation time and incubation temperature,

and concentration of the antimicrobial compound. The results has been interpreted based on the susceptibility and resistance according to the presence or absence of a zone of inhibition surrounding the disk, and two or three different concentrations of the same antimicrobial will be routinely tested against the pathogen. In 1956, W. M. M. Kirby and his colleagues at the University of Washington School of Medicine and the King County Hospital proposed a single disk method for antimicrobial susceptibility testing. Later, Kirby and his colleague, A. W. Bauer, extensively studied this method and consolidated and updated all the previous setbacks of the disk diffusion method and published their findings. The World Health Organization to form a committee in 1961 to review the protocol for single antimicrobial disk susceptibility testing. The result was a standardized procedure for the disk diffusion assay called the Kirby-Bauer disk diffusion test.

At present, the Clinical Laboratory Standards Institute (CLSI) is updating and modifying the original procedure of Kirby and Bauer throughout a global requirement and developing a standard technique with highest reproducibility of results as pathogens develop new mechanisms of resistance and new antimicrobials are developed to fight these organisms. The CLSI approved Standards for Antimicrobial Disk Susceptibility Tests have been used in all clinical laboratories today.

14. 2 OBJECTIVES

After going through this Experiment you will be able to;

- Perform Kirby-Bauer disk diffusion [antimicrobial impregnated filter paper disks] susceptibility test to determine the sensitivity or resistance of pathogenic bacteria to various antimicrobial compounds.
- Understand the CLSI standards and its importance.

14. 3 ABOUT THIS EXPERIMENT

Kirby and Bauer Method

Determination of bacterial sensitivity or resistance to antimicrobials is an important part of the treatment to infectious diseases. The standard disk diffusion method of Kirby and Bauer has been a viable alternative to broth dilution methods. When a filter paper disk (6 mm) impregnated with a known concentration of an

antimicrobial compound is placed on a pathogen-inoculated Mueller-Hinton (MH) agar plate, instantly water is absorbed into the disk from the agar. The impregnated antimicrobial agent begins to diffuse into the surrounding agar. The concentration of antimicrobial is high near the disk surrounding and low at the distance from the disk increases. The diffusion rate of the antimicrobial through the agar is dependent on the solubility of the drug in MH agar and the molecular weight of the antimicrobial compound. Low molecular weight compound diffuses faster than the larger molecules. Combination of the above factors, result in each antimicrobial having a unique division zone size representing susceptibility to that antimicrobial compound.

If the MH agar plate has been inoculated with a suspension of the test pathogen prior to placing of disks on the agar surface, simultaneously diffusion of the antimicrobial compounds and growth of the bacteria could occurs. Growth occurs in the presence of an antimicrobial compound when the bacteria reach a critical mass and can overpower the inhibitory effects of the antimicrobial compound. The estimated time of a bacterial suspension to reach critical mass is 8 to 12 hours (37 ^{o}C) for most common pathogens. The size of the inhibition zone is influenced by the depth of the agar. The antimicrobial compound diffuses in three dimensions; hence a shallow layer of agar will produce a larger zone of inhibition than a dense layer. The point at which critical mass is reached is demonstrated by a sharp margin circle of bacterial growth around the disk. The concentration of antimicrobial compound at this margin is called the crucial concentration and is approximately equal to the minimum inhibitory concentration obtained in broth dilution susceptibility tests. The size of the inhibition zone is correlated with the interpretive standards (CLSI, 9th Edition).

McFarland standard

McFarland standards are suspensions of either barium sulfate or latex particles that allow visual comparison of bacterial density (Fig.14.1). Commercially prepared standards are available for purchase from companies such as Remel or BD BBL. These often include a Wickerham card, which is a small card containing parallel black lines. A 0.5 McFarland standard is equivalent to a bacterial suspension containing between 1 x 10^8 and 2 x 10^8 CFU/ml of *E. coli*.



Fig.14.1: McFarland standards.

14.4 MATERIALS REQUIRED

1. Sterile saline in 2-ml tubes
2. 0.5 McFarland standard
3. Wickerham card
4. Mueller-Hinton agar plates
5. Wickerham card
6. Ruler for measuring zone of inhibition
7. Antibiotic disks
8. Forceps (or) Antibiotic disk dispenser
9. Vortex
10. Sterile swabs
11. Inoculating loop
12. Alcohol pads
13. Incubator [35°C to 37°C]
14. Test organisms: *Staphylococcus aureus* ATCC 25923; *Escherichia coli* ATCC 25922; and *Pseudomonas aeruginosa* ATCC 27853.

14. 5 PROCEDURE

14.5.1 McFarland standard [0.5] Preparation

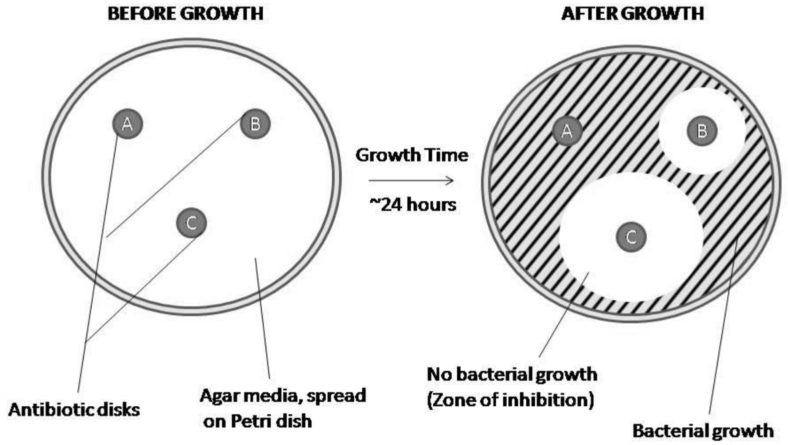
1. Add a 0.5-ml aliquot of a 0.048 mol/liter $BaCl_2$ (1.175% wt/vol $BaCl_2 \cdot 2H_20$) to 99.5 ml of 0.18 mol/liter H_2SO_4 (1% vol/vol) with constant stirring to maintain a suspension.
2. Verify the correct density of the turbidity standard by measuring absorbance using a spectrophotometer with a 1-cm light path and

matched cuvette. The absorbance at 625 nm should be 0.08 to 0.13 for the 0.5 McFarland standard.

3. Transfer the barium sulfate suspension in 4- to 6-ml aliquots into screw-cap tubes of the same size as those used in standardizing the bacterial inoculums.
4. Tightly seal the tubes and store in the dark at room temperature.

14.5.2 Antibiotic Sensitivity Assay

1. Prepare Mueller-Hinton plates.
   a. Prepare MH agar medium and pour in Petri plates and allowed to solidify without any air bubbles.
   b. Appropriately label each MH agar plate for each organism to be tested.
2. Prepare bacterial inoculums of the test organisms as per the CLSI standard.
   a. Using a sterile inoculating loop or needle, scrap four or five pure identical isolated colonies of the organism to be tested.
   b. Suspend the organism in 2 ml of sterile saline.
   c. Vortex the saline tube to create a smooth suspension.
   d. Adjust the turbidity of this suspension to a 0.5 McFarland standard by adding more organism if the suspension is too light or diluting with sterile saline if the suspension is too heavy.
   e. Use this suspension within 15 minutes of preparation.
3. Inoculate the test organisms in the MH agar plates.
   a. Dip a sterile swab into the inoculum tube.
   b. Rotate the swab against the side of the tube (above the broth) using solid pressure, to remove excess fluid. The swab should not be dripping wet.
   c. Inoculate the dried surface of a MH agar plate by streaking the swab over the entire agar surface to ensure an even distribution of the inoculum (Fig. 14.2).
   d. Rim the plate with the swab to pick up any excess liquid.
   e. Discard the swab into an appropriate container.
   f. Leaving the lid slightly ajar, allow the plate to sit at room temperature (up to 3 minutes) for the surface of the agar plate to dry before introducing the antibiotic disc.



4. Place the appropriate antimicrobial-impregnated disks on the surface of the agar (fig.14.2).

a. Using either forceps or a disc dispenser to dispense each antimicrobial disk one at a time.

b. Place the dispenser over the culture inoculated MH agar plate and firmly press the plunger once to dispense the disks onto the surface of the plate. Repeat this procedure to place all antibiotic disc on the MH agar plate with subsequent space (minimum 3 cm distance). **(Or)** Sterilize the forceps by cleaning them with a sterile alcohol pad and allowing them to air dry. Using the forceps carefully remove one disk from the cartridge and place the disk on the plate with subsequent space (minimum 3 cm distance) and gently press the disk with the forceps to ensure complete contact with the agar surface.

c. Do not move a disk once it has contacted the agar surface even if the disk is not in the proper location, because some of the drug begins to diffuse immediately upon contact with the agar.

d. Close the agar plate, invert the plates immediately.

5. Incubate the MH agar plates in a 35°C ± 2°C air incubator for 16 to 24 hours.

6. Following incubation, measure the zone sizes to the nearest millimeter using a ruler or caliper; include the diameter of the disk in the measurement (Fig. 2).

a. When measuring zone diameters, always round up to the next millimeter.

b. All measurements are made with the unaided eye while viewing the back of the petri dish. Hold the plate a few inches above a black, nonreflecting surface.

c. Distinct, discrete colonies within an obvious zone of inhibition should not be considered swarming. These colonies are either

mutant organisms that are more resistant to the drug being tested, or the culture was not pure and they are a different organism.

d. Record the zone size on the recording sheet.

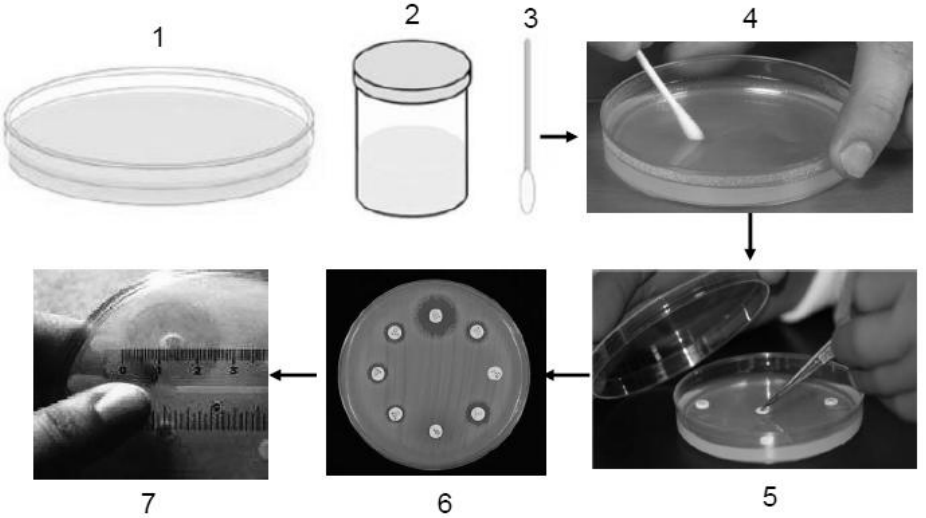


Figure -14.2: Steps for antibiotic sensitivity test. 1) Sterile MHA plate, 2) sensitive bacterial culture, 3) sterile bud, 4) swabbing, 5) placing antibiotic disc, 6 &7 observation and zone diameter measurement.

14.6 OBSERVATION & RESULT

Antibiotic Sensitivity test

S.No.	Antibiotic disc	Concentration	Zone diameter (mm)		
			E. coli	*S. aureus*	*P. aeruginosa*
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					

Interpretation [Susceptible (S), intermediate (I), or resistant (R)]

NOTES

14.7 LET US SUM UP

In this experiment, antibiotic sensitivity of the bacteria has been determined. Through this method, sensitivity, intermediate resistant and resistant based classification of microbes against the particular antibiotic has been determined.

14.8 UNIT - END EXERCISES

1. Discus Kirby Bauer's method.
2. What are McFarland standards?
3. How do you classify the bacteria based on the results of antibiotic sensitivity results?

14.9 SUGGESTED READING

1. Microbiology- A laboratory Manual-International student Edition: 4th edition. By James G. Cappuccino& Natalie Sherman
2. Bacteriological Techniques By F.J. Baker
3. Introduction to Microbial Techniques By Gunasekaran
4. Manual of Industrial Microbiology and Biotechnology, 2nd Edition by Arnold; . Demain and Julian, E. Davie Ronald M. Atlas, Gerald Cohen, Charles L. Hershberger, Wei-Shou Hu, David H. Sherman, Richard C. Wilson and J. H. David Wu.

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UNIT – 15: MIC AND MBC DETERMINATION

Structure

15.1 INTRODUCTION

The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that prevents the visible growth of bacteria (bacteriostatic). MICs are used to assess the antimicrobial efficacy of a variety of compounds. It could be determine the effect of decreasing concentrations of antibiotic on the growth inhibition over a defined time period. The MIC evaluations can be quite useful to treat the infectious disease as well as to determine the appropriate concentrations of the required drug in the final product during the R&D phase of pharmaceuticals. It will reduce the overuse defects (side effects) to the patients.

For the study of MIC, various concentrations of the compounds are inoculated with cultured bacteria, and the results are measured using agar dilution or broth dilution (macro or micro) to determine at what range the MIC endpoint is developed. Susceptibility testing is typically conducted using organisms that contribute to an infectious process demanding antimicrobial chemotherapy. The antimicrobial resistance of the common nosocomial (hospital-related) infections bacteria referred as the ESKAPE pathogens which include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species have been frequently detected by this method.

The Minimum Bactericidal Concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a bacterium over a fixed, somewhat extended period, such as 18 hours or 24 hours, under a specific set of conditions. It can be determined from the broth dilution of MIC tests by sub-culturing to agar plates that do not contain the test agent. The MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by a pre-determined reduction such as ≥99.9%. The MBC is complementary to the MIC; whereas the MIC test demonstrates the lowest level of antimicrobial agent that greatly inhibits growth, the MBC demonstrates the lowest level of antimicrobial agent resulting in microbial death. In other words, if a MIC shows inhibition, plating the bacteria onto agar might still result in organism proliferation because the antimicrobial did not cause death. Antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC. MBC testing can be a good and relatively inexpensive tool to simultaneously evaluate multiple antimicrobial agents for potency.

15. 2 OBJECTIVES

After going through this Experiment you will be able to;

- Perform minimum inhibitory concentration of an antibiotic.
- Evaluate the minimum bacteriocidal and static concentration of an antibiotic.

15. 3 ABOUT THIS EXPERIMENT

In this experiment, to examine systematically the effects of a selective antibiotic at different concentration level on the sensitive bacteria. It usually carried out for both Gram-positive and Gram-negative bacteria. Dilution methods include broth macrodilution, broth microdilution, and agar dilution. Conventional broth microdilution methods based on the Clinical and Laboratory Standards Institute (CLSI) have been used to evaluate the antimicrobial activity for both bacteria and fungi. In this method of broth microdilution, twofold dilutions of antimicrobials are made in a broth medium in a microtiter plate (Figure 15.1) [96-microtiter well plate] that expresses the results in micrograms per milliliter (µg/mL) to identify the MIC that is the lowest concentration by visual observation of an antimicrobial agent that inhibits the growth (Figure 15.1) of tested bacteria/fungi. Dilutions can also be performed in agar, each dilution being poured onto a plate

in a standardized fashion and allowed to set before inoculating it with the organism(s) of interest. Agar dilution can be used to perform susceptibility testing of fastidious bacteria with special medium requirements. These methods have the limitation that the MIC alone does not provide the mechanism of action of the antimicrobial; for that motive, each antimicrobial protocol should be complemented using static or cidal activity.

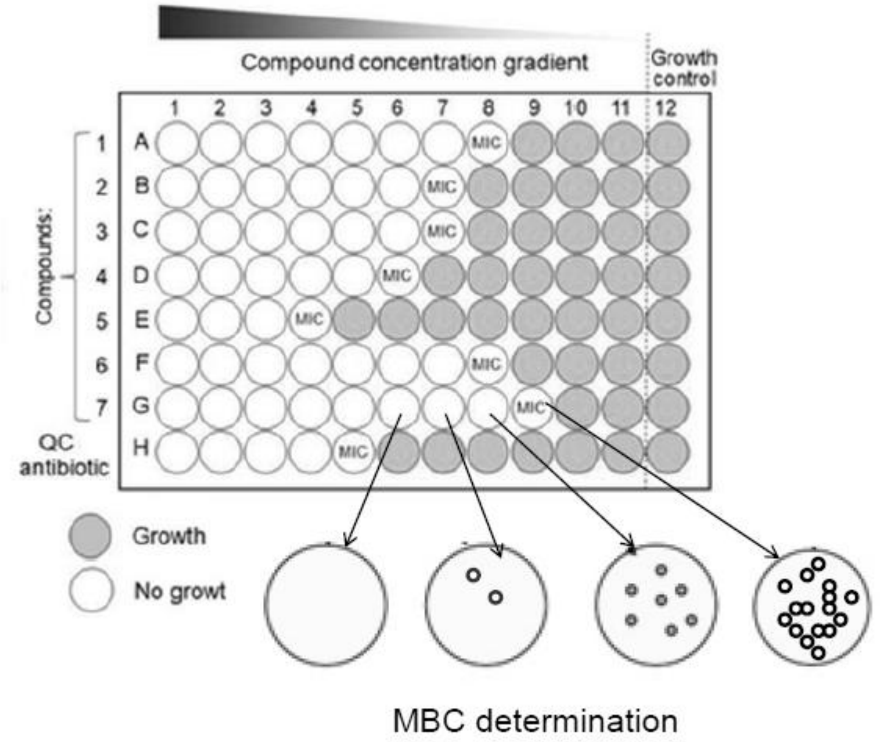


Figure-15.1: MIC and MBC determination.

15.4 MATERIALS REQUIRED

1. Microtitre plates
2. Sterile nutrient broth
3. Mueller-Hinton agar plates
4. Antibiotic stock solutions (100µg/mL)
5. Micropipette
6. Incubator [35°C to 37°C]
7. Test organisms: *Staphylococcus aureus* ATCC 25923; *Escherichia coli* ATCC 25922; and *Pseudomonas aeruginosa* ATCC 27853.

15. 5 PROCEDURE

15.5.1 MIC determination [microdilution assay]

1. A single 96-well microdilution plate can be used for up to 4 drugs.
2. Each drug should be tested in duplicate. Label the wells as; rows A, B (Drug 1); rows C, D (Drug 2); rows E, F (Drug 3); and rows G, H (Drug 4).
3. Wells 1–8 is to test the number of dilutions necessary for an end point MIC. Use wells 9, 10, 11, and 12 for solvent, media, drug, and growth controls, respectively.
4. Add 0.025 ml of appropriate broth medium in wells 2–8, 10, and 12 of the microdilution plate.
5. Add 0.025 ml of the highest concentration of drug (100µg/ml) solution to be tested to wells 1 and 11 in rows A and B. Well 11 will serve as the drug control. The other drugs to be tested will be added the same way in their respective rows.
6. Dilute the antimicrobial agent in serial two-fold dilutions using a 0.025-ml micro pipette, beginning at the second well and continuing through well 8. Discard the final 0.025 ml of antimicrobial agent solution.
7. Prepare a solvent control in well 9 by incorporating 0.025 ml of a 1:10 dilution of the solvent used to dissolve the antimicrobial agent being tested [usually alcohol/methanol or dimethyl sulfoxide (DMSO) are used as solvents].
8. Prepare the test organisms into 10^4–10^5 CFU/ml in the appropriate broth medium and pre-incubate for 2 hours. Add 0.175 ml of the desired dilution of the organism inoculum to each well 1–9, 11, and 12. Well 12 serves as the growth control. Add 0.175 ml of the appropriate uninoculated medium to wells 10 and 11 (total of 0.2 ml) for media and drug controls.
9. Incubate microdilution plates at 37°C in an ambient air incubator for 12 to 24 hours.
10. Read the microdilution plates after 18–24 hours of incubation at 590nm using microplate reader.
11. Record the MIC as the concentration of antimicrobial agent inhibiting visible color change in broth medium wells (wells 1 through 8), well 12 is consider as the organism control positive.
12. Report the highest end point/ lowest drug inhibits visible growth inhibition, as MIC of the drug agaist te bacteria.

Note: *Control wells and expected results: well 9 (i.e., solvent control) – no color change; well 10 (i.e., media control) – no color change; well 11 (i.e., drug control) – no color change; well 12 (i.e., growth control) – growth and color change according to organism being tested, without turbidity.*

15.5.2 MBC Determination [Fig. 15.1]

1. To determine the MBC, the dilution representing the MIC and at least two of the more concentrated test product dilutions are plated and enumerated to determine viable CFU/ml.
2. 50 µL of the MIC and other two lowest concentrations will be plated on Nutrient agar/Muller Hinton Agar plates [spread plate method].
3. Incubate the plates at 37^{o}C for 24 hours.
4. Observe the CFU, count the total CFU/plate.
5. Calculate the MBC: the lowest concentration that demonstrates a pre-determined reduction (such as 99.9%) in CFU/ml when compared to the MIC dilution.

13.6 OBSERVATION & RESULT

Table : MIC and MBC of bacteria A & B

S.No.	**Antibiotic concentration**	Growth at row		MIC value	MBC value
		A	B		
1	250				
2	125				
3	67.5				
4	33.75				
5	16.88				
6	8.42				
7	4.21				
8	2.1				

Antibiotic Sensitivity test (Growth +; no growth -) (or) OD at 590nm

Interpretation

NOTES

15.7 LET US SUM UP

In this experiment, you have learnt about the MIC and MBC of the particular antibiotic against the target pathogen. The principle, method and the results evaluations also studied. In addition, the method of microdilution also learnt.

15.8 UNIT - END EXERCISES

1. Discus microdilution method.
2. Differentiate MIC and MBC?
3. How do you determine the MIC and MBC of an antibiotic against the target pathogen?

15.9 SUGGESTED READING

1. Microbiology- A laboratory Manual-International student Edition: 4th edition. By James G. Cappuccino& Natalie Sherman
2. Bacteriological Techniques By F.J. Baker
3. Introduction to Microbial Techniques By Gunasekaran
4. Manual of Industrial Microbiology and Biotechnology, 2nd Edition by Arnold; Demain and Julian, E. Davie Ronald M. Atlas, Gerald Cohen, Charles L. Hershberger, Wei-Shou Hu, David H. Sherman, Richard C. Wilson and J. H. David Wu.
5. Jennifer M. Andrews. Determination of minimum inhibitory concentrations Journal of Antimicrobial Chemotherapy (2001) 48, Suppl. S1, 5–16

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UNIT – 16: SEPARATION OF PROTEIN

Structure

16.1 INTRODUCTION

Protein separation and purification is a series of processes intended to isolate one or few proteins from a complex mixture, usually from cells or tissues or whole organisms. Protein purification is important for the characterization of the proteins structure, function, and its interactions of interest. The purification process may separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious aspect of protein purification. Separation steps usually depend on the protein size, physico-chemical properties and biological activity.

If the targeted protein is not in secreted form, the first step of purification process is the disruption of the cells containing the protein. Many methods have been available for cell lysis, which includes, i) homogenization, ii) sonication, iii) repeated freezing and thawing, and iv) permeabilization by detergents (e.g. Triton X-100) and/or enzymes (e.g. lysozyme). After cell lysis, the cell debris can be removed by centrifugation so that the proteins and other soluble compounds remain in the supernatant. If purification of integral membrane proteins requires disruption of the cell membrane using mild detergents like Triton X-100 or CHAPS instead of using sodium dodecyl sulfate (SDS) which causes denaturation.

For the protein purification, after the clarification a common first step to isolate proteins is precipitation with ammonium sulfate $(NH_4)_2SO_4$. Protein precipitation is achieved by adding increasing amounts of ammonium sulfate and collecting the different fractions of precipitate protein. Once the protein is precipitated, ammonium sulfate can be removed by dialysis. The concentrated protein is used for further purification by ion-exchange chromatography, Gel filteration chromatography and finally purity of the protein will be analyzed by Poly Acrylamide Gel Electrophoresis (PAGE).

16. 2 OBJECTIVES

After going through this Experiment you will be able to;

- Perform an experiment to prepare cell lysate for protein separation.
- Concentrate the protein by Ammoniam Sulphate precipitate (salting out) method.
- Perform the experiment to remove excess salt by dialysis.
- Separate the proteins by Ion-exchange chromatography.
- Perform paper chromatography for separation of amino-acids.

16. 3 ABOUT THIS EXPERIMENT

16.3.1 Cell Lysate Preparation

Preparation of samples for protein analysis requires two essential steps: disruption of the cells to cellular materials. Mechanical homogenization and sonication (ultrasonic homogenization) are two techniques used for these processes.

Mechanical homogenization utilizes direct physical force to bring microbial cells in solution to a state of uniform distribution, such that all fractions' molecular composition is consistent. Traditionally, mechanical disruption was achieved by freezing the cell biomass/ tissues and then grinding with a mortar and pestle. Manual grinding is the most common method used to disrupt plant cells. Cell mass /tissue is frozen in liquid nitrogen and then crushed using a mortar and pestle. This method is the fastest and most efficient way to access proteins and DNA. In addition, sonication is the act of applying sound energy to agitate particles in a sample, for various purposes such as the extraction of multiple compounds from plants, microalgae and seaweeds. Ultrasonic frequencies (>20 kHz) are usually used, leading to the process also being known as ultrasonication or ultra-sonication. Presently, there are two other common methods: bead-based disruption and rotor-stator disruption. These separation techniques efficiently homogenize samples, but often require suitable downstream fractionation to obtain the desired concentration or purity of molecules. In this experiment, harvested bacterial cells are frozen and grind with liquid nitrogen using mortar and pestle and alternatively by sonication.

16.3.2 Ammonium Sulphate precipitation of proteins

Ammonium sulfate precipitation is one of the most commonly used methods for protein purification from a solution. In solution, proteins form hydrogen bonds with water molecules through their exposed polar and ionic groups. When high concentrations of small, highly charged ions such as ammonium sulfate are added, these groups compete with the proteins to bind to the water molecules. This removes the water molecules from the protein and decreases its solubility, resulting in precipitation. Critical factors that affect the concentration at which a particular protein will precipitate include: the number and position of polar groups, molecular weight of the protein, pH of the solution, and temperature at which the precipitation is performed.

16.3.3 Protein Dialysis

Dialysis is a commonly used laboratory-scale process to remove salt, or reduce the salt concentration, from a solution. A semi-permeable membrane is used to contain the target protein. The target protein in solution is placed into the dialysis tubing and the dialysis

tubing is sealed and placed into a large container of water (Fig. 2). The salt in the dialysis tubing moves from high concentration to low concentration via passive diffusion. The final salt concentration in the protein solution is the weighted average of the salt concentration based on the volumes of the starting protein solution and the water. As the volume of the solution in the dialysis tubing decreases, the protein concentration increases. Continuous dialysis is called diafiltration, and the protein solution is diluted with large amounts of water and then passed through an ultrafiltration device to remove both the salts and water. Both diafiltration and dialysis methods can be repeated by adding or contacting, respectively, the target solution with more water. There are a lot of coefficients that affects the efficiency of dialysis, such as pH, Temperature, Time, Buffer solution volume and Thickness of dialysis membrane.

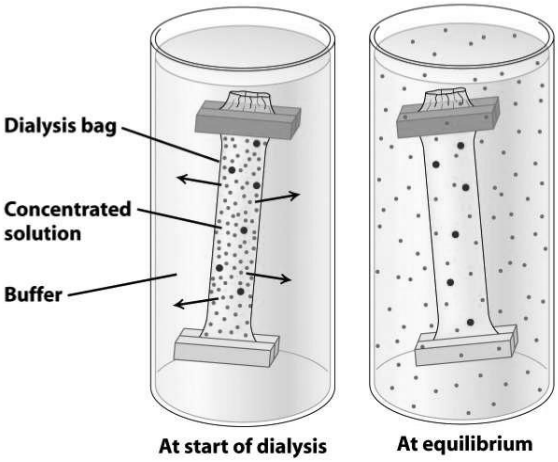


Figure -16.1: Method of dialysis.

16.4 MATERIALS REQUIRED

1. LB medium
2. *E.coli*
3. Lysis buffer: 50mM Tris pH 8.0
4. Liquid nitrogen, mortar and pestle
5. Ammonium Sulphate
6. Centrifuge
7. Dialysis Membrane (10 kDa)
8. Protein concentrate
9. Dialysis buffer.
10. Distilled water

16. 5 PROCEDURE

16.5.1 Cell Lysis preparation

1. Grow starter from a single colony on LB medium.
2. Incubate the cells for 24 hours at 37^0C.
3. Harvest cells by centrifugation (8000 rpm/5min), aspirate supernatant and freeze pellet at -70^{o}C.
4. Resuspend 1.5 ml pellet of bacterial cell culture in 0.75 ml of lysis buffer.
5. Incubate on 30ºC 15 minutes or 30min on ice.
6. Add equal volume of liquid nitrogen and grind in mortar and pestle to make fine bacterial homogenate.
7. Collect the lysate into a new tube, centrifuge the content at 8000 rpm for 10 min at 4^{o}C.
8. Collect the supernatant for further processing.

Sonication

1. Follow the steps 1 to 5.
2. Sonicate 3x20'' till sample is no longer viscous.
3. Place the tube on ice and immerse probe in the sample.
4. Press the Start key and pulse 3 times 30 seconds for each sample, until sample gets clear. Place the probe inside the ice for 20 seconds between samples. (For prevention of sonicator over-heating!) [Press Pulser: Cycle ON 5 seconds, cycle OFF 5 seconds]

[Note: While sonicating, make sure sample is not getting hot as the sonication proceeds]

5. Centrifuge 10,000rpm for 10min at 4^{o}C.
6. Collect supernatent to new tube for further process.

16.5.2 Ammonium Sulphate Precipitation

1. Allow the cell lysate [supernatant] to thaw, determine total volume, and centrifuge at 3000g for 30 minutes.
2. Transfer sample to a beaker containing a stir bar and place on a magnetic stirrer.
3. While the sample is stirring, slowly add saturated ammonium sulfate to bring the final concentration to 50% saturation.
   i. Volume of ammonium sulfate needed is equal to the volume of sample.

ii. Adding the ammonium sulfate very slowly ensures that local concentration around the site of addition does not exceed the desired salt concentration.

4. Once the total volume of ammonium sulfate is added, move beaker to 4°C for 6 hours or overnight.
5. Transfer to conical tube and centrifuge the precipitate at 3000g for 30 minutes.
6. Carefully remove and discard supernatant. Invert conical tube and drain well. For cell lysate protein, resuspend the pellet in 30%-50% of the starting volume in 1XPBS.
7. Determine the protein concentration and store at -80°C for long-term storage.
8. Tubing and dialyze versus three changes of 1XPBS/0.08% Sodium Azide. Be sure to allow enough space for expansion of the antibody solution during dialysis. Normally twice the re-suspended volume is sufficient.
9. Remove antibody solution from the tubing and centrifuge to remove any remaining debris.

16.5.3 Dialysis

1. After cutting the proper length of the dialysis bag, soak in dialysis buffer
2. Clip the bottom of the dialysis bag with metal clip to seal well. Add the protein into the dialysis bag, and get rid of all bubbles. Clip the other end with plastic clip and tie an eppendorf on to it.

[Note: *The dialysis bag should be suspende din the dialysis buffer, thus the two side of the dialysis bag would be clipped with different kind of clips*].

3. Put a stir bar in the dialysis buffer and stir at 4^oC

[Note: *Stir bar would attract the metal clip by magnetic force; the dialysis bag would rotate with the stir bar*].

4. Change dialysis buffer every 3 hours. The last stage of dialysis can be stored overnight.
5. Collect samples from dialysis bag.
6. Centrifuge13000 rpm, 4^oC, 20mins.
7. After centrifuging, obtain the supernatant and store at -20^oC.
8. Estimation of the protein content.

16.6 ION-EXCHANGE CHROMATOGRAPHY

16.6.1 Principle

Ion- exchange chromatography is based on electrostatic interactions between charged protein groups, and solid support material (matrix). Ion-exchange chromatography is governed by the principles of ionic chemical interactions that lead to reversible adsorption of the analyte on the stationary phase material. The strength of the binding between the analyte and the column matrix is determined by the functional groups on the sample (anlyte) and the resin (matrix).Two types of resins are used. Strong-cation exchange resins typically feature sulfonic acid functional groups while weak cation-exchange resins have carboxylic acids. Strong anionic-exchange resins contain quaternary amines while weak anion-exchange resins feature secondary or tertiary amines. The terms strong and weak refer to the acid/base properties of the column material and not how well it binds an analyte. Weak resins are charged over a smaller pH range than strong resins, but still bind the analytes very effectively, and could be a good choice if they are charged in the pH range needed. Before use, ion exchange columns are equilibrated at a pH using an equilibration buffer.

The elution buffers always salt solutions, it depends on the sample of interest, a linear gradient of salt solution is used. Samples that are less tightly bound to the resin will elute first, as the salt will most easily disturb their ionic bonding to the column. Samples that are more tightly bound will elute later, when higher amounts of salt are used. However, step gradients can also be used where the concentration of salt is stepped up over time.

pH of the buffers is the important consideration for ion-exchange chromatography. The pH needs to be maintained so that the analyte of interest is charged and will bind to the resin. For proteins, the charge is based on the protein's isoelectric point, where the protein is neutrally charged, and measured as pI. The pH in comparison to the protein pI gives information about the expected protein charge. In cation-exchange chromatography, raising the pH will cause the analyte to be less positively charged and less likely to interact with the negatively-charged resin. Similarly, in anion-exchange chromatography, lowering the pH will cause the analyte to be less negatively-charged and less likely to interact with the positively-charged resin. Thus pH adjustments

can also be used to selectively elute analytes from the column. The use of pH adjustments instead of salts could be advantageous for separating two different types of proteins with different pI values.

In this experiment, negatively charged protein like α-amylase enzyme protein will be purified using DEAE-Cellulose column (anion exchanger). Gel **matrix** beads are derivatized with diethylaminoethanol (**DEAE**) and lock negatively charged proteins. DEAE-cellulose contains an diethylaminoethyl group. It is positively charged at neutral pH and so DEAE-cellulose is a weak anion exchanger. DEAE-cellulose begins to lose its charge above pH 9. One can elute the bound proteins by increasing ionic strength irrespective of whether an anion or cation exchanger was used. This is usually accomplished by incorporating a linear concentration gradient of NaCl in the column buffer while keeping the pH constant.

16.6.2 MATERIALS REQUIRED

1. Column tube – 10 ml or 20 ml capacity
2. DEAE-Cellulose
3. NaCl
4. Sample buffer (pH 7.0)
5. Column Activation Buffer (pH 7.0)
6. Test tubes, Spectrophotometer, etc.

16.6.3 PROCEDURE

1. Preparing the Sample and the Column
   1. Alpha amylase from bacillus extracellular protein concentrate is used. Add 0.2 ml of equilibration buffer (pH 7.0) to the protein sample and vortex to mix thoroughly.
   2. Place the anion-exchange column in a glass column tube (1 x 20 cm) for 5 min to allow resin to settle (5ml volume). Fix the column in upright position.
   3. Open the top cap of the column, and then the bottom cap. Allow the buffer in the column to drip out under gravity into the test tube.
   4. Wash the column twice with a column-volume (in this case the column volume is 5ml mL) of equilibration buffer. Let the column-volume (5.0 ml) of equilibration buffer drip out into the waste vial.

2. Running a Protein Sample Through a Anion-Exchange Column
   1. Carefully load 0.5 mL of the protein sample onto the top of the column.
   2. Once the sample has been loaded onto the top of the column, wash it with 2 mL of equilibration buffer by adding the buffer at the top of the column and allowing it to drip all the way through. Repeat this step 2x (for a total of 3 washes) and collect each wash in a separate. Label the tubes as 1-3.

   [Any sample that binds to the column should not be in these washes, but sample that does not bind will wash out unretained].

   3. Put 2.0 mL of elution buffer (high salt, pH 7.5) on top the column. Collect the drain.
   4. Repeat the elution step 3 more times to collect the elutions and named the tubes as 4-n (number of repeats).
   5. Take OD value at 280 nm in spectrophotometrically against the elution buffer blank.
   6. Check the enzyme activity of the individual fractions and record the results

16.6.4 BRADFORD PROTEIN ASSAY

16.6.4.1 Principle

The Bradford Protein Assay measures the concentration protein by adding Coomassie dye to the sample under acidic conditions. When proteins bind with the Coomassie dye, the sample changes color from brown to blue. The level of blue can then be measured using a spectrophotometer to determine the concentration of protein in the sample. Technically the Bradford Protein Assay is only measuring the basic amino acids, arginine, lysine, and histidine. However, most proteins have a fairly balanced level of these amino acids with all other amino acids, so we can still extrapolate the level of total protein in the sample. But, if a sample is particularly high or low in any of these amino acids, then this is not the right test to use.

16.6.4.2 MATERIALS REQUIRED

BSA standard (100µg/ml)
Coomassie dye
Spectrophotometer, curettes, Pipettes, Test tube
Protein sample

16.6.4.3 PROCEDURE

a) Standard

For the standard, you can use any complete protein, but typically **bovine serum albumin** (BSA) is used as the standard, since it is cheap and easy to come by.

1. Prepare several dilutions of the BSA standard, at least 5. For example, the dilutions may be 5, 10, 25, 50, 75, and 100 micrograms of BSA per milliliter.
2. Add reagent (which contains an acid and the Coomassie dye) to the BSA dilutions
3. Incubate for 5 min to 1 hour
4. Measure absorbance, with spectrophotometer set at 595 nm

The data obtained here can be used to create a graph, with the absorbance on the y-axis and the known protein concentration on the x-axis. A fairly linear line should be able to be drawn between each of the points, and the equation of the line determined.

b) Sample

1. Dilute sample so that it falls within the BSA standard curve
2. Add Bradford reagent
3. Incubate for 5 min to 1 hour (as close as possible to how long the BSA was incubated)
4. Measure absorbance, with spectrophotometer set at 595 nm

16.6.5 OBSERVATION AND RESULT

Each fraction from the ion-exchange column should be used as protein source for protein quantification. Quantity of the protein will be estimated by **Bradford Protein Assay.**

Protein Standard Graph

Si No	Protein Concentration [µg/mL]	OD value
1	5	
2	10	
3	25	
4	50	
5	75	
6	100	

Protein Quantity in fractions of Ion-exchange chromatography

Fraction No	Protein Qty.	Fraction No	Protein Qty.	Fraction No	Protein Qty.

Result:

The maximum quantity of protein is extracted in the fraction No.________ .

Note: *Based on the activity, the expected protein fraction will be determined by selecting suitable protocol*.

16.7 PAPER CHROMATOGRAPHY METHOD

16.7.1 PRINCIPLE

Paper chromatography has proved to be very successful in the analysis of chemical compound and lipid sample in particular. In paper chromatography, the sample mixture is applied to a piece of filter paper, the edge of the paper is immersed in a solvent, and the solvent moves up the paper by capillary action. It is the simplest and commonest form of liquid-liquid chromatography.

In 1944, Consden, Gordon, and Martin describe the basic principle of this procedure. Components of the mixture are carried along with the solvent up the paper to varying degrees, depending on the adsorbed compounds soluble in the solvent used. Whatman filter paper is used for this process. The paper is composed of cellulose to which polar water molecules are adsorbed, while the solvent is less polar, usually comprising a mixture of water and an organic liquid.

The paper is called the stationary phase while the solvent is referred to as the mobile phase. To get a measure of the extent of movement of a component in a paper chromatography experiment, we can calculate an "Rf value" for each separated component in the developed chromatogram. An Rf value is a number that is defined as the distance traveled by the component from an application point. There are four main techniques, which may be employed for the development of paper Chromatograms. They are, Ascending techniques, Descending techniques, Radial development and Two-dimensional chromatography.

16.7.2 Materials Required

1. Support for paper
2. Airtight chamber
3. Whatman filter paper number 1
4. Capillary tubes
5. Samples–Amino acids (or) Pigments
6. Solvents: **B**utanol: **A**cetic acid: **W**ater (4:1:5),
7. Platinum loop
8. Ninhydrin solution (0.5% in acetone), ninhydrin sprayer.
9. UV-chamber

16.7.3 PROCEDURE

1. Take a filter paper What man No.1, and cut in to 8 x 12 cm size.
2. A horizontal line is drawn near one end (about 1.5 cm from the bottom edge) of the paper.
3. The sample needs to be separated is placed as a small drop on to the paper using capillary tube.
4. Labeling the drop by a pencil with an alphabet or number help to identify the compound later.
5. The drops are then soaked on the paper and dried.
6. The paper is then placed into a sealed container with a swallow layer of suitable solvent.
7. The solvent level must be lower than the pencil line or drop on it. The container needs to be covered to stop the solvent to evaporate.
8. The solvent rises up the paper chromatography taking each component of the sample with it. The components travel with the solvent depends on three things:

- The polarity of the sample molecule. The non polar components travel faster than the polar component.
- The attraction between the sample molecule and the solvent or solvent mixture.
- The attraction between the sample and the silica.

9. When the solvent rises near the end of the paper then the paper should be taken out from sealed container and air dried.
10. The paper with separated bands of components is then observed under UV-light.
11. Spray with ninhydrin solution. Allow to dry in oven (80 – 100 ^{o}C). Observe the colour development.

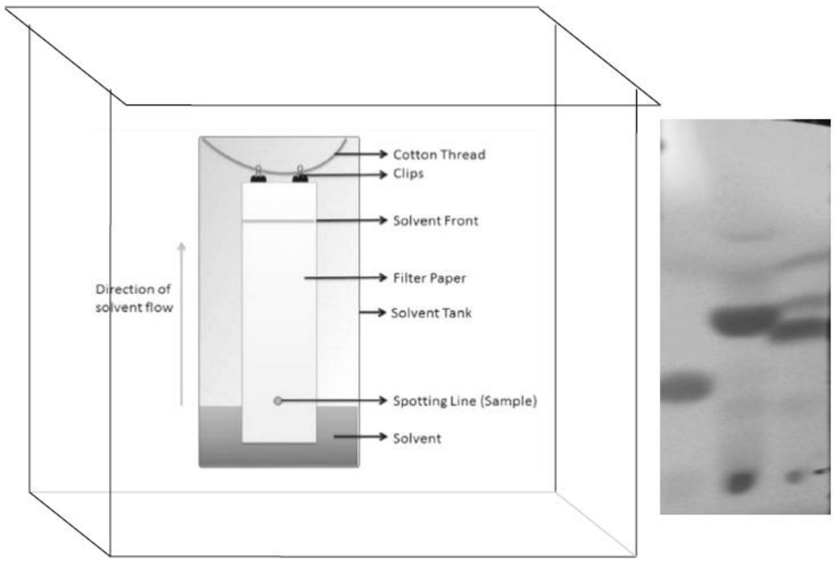
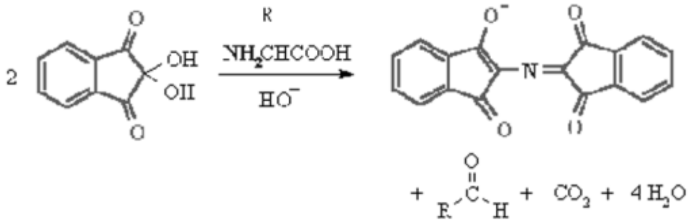


Figure 1: Paper chromatography technique.

Ninhydrine Reaction: The filter paper strip may be sprayed with ninhydrin and heated so that the colored spots showing the location of amino acids may develop. The color densities of these spots may be measured with a recording (or) reflectance photometer device. Amines (including α-amino acids) react with ninhydrin to give a colored product. It can be used qualitatively (*e.g.* for chromatographic visualization) or quantitatively (*e.g.* for peptide sequencing). The α-amino acids typically give a blue-purple product. Proline, a secondary amine, gives a yellow-orange product. The test is sensitive enough that ninhydrin can be used for the visualization of fingerprints.



R_f value calculation: The compounds in the sample travels along with solvent to give separate bands on the paper. The distance travelled by same compound with respect to the solvent is always constant. Thus the ratio of the distance that the compound travelled and the distance that the solvent travelled is denoted as R_f. And mathematically expressed as:

Rf = Distance travelled by the solute / Distance traveled by the solvent

16.7.4 OBSERVATION

Observations can be recorded as shown.

SI No.	Components	Distance travelled by the component from the original line (cm)	Distance travelled by the solvent from the original line (cm)	R_f value
1	Spot-1			
2	Spot-2			
3	Spot-3			

Interpretation

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16.8 LET US SUM UP

In this experiment, you have learnt about the protein preparation based on the cell lysis, ammonium sulphate precipitation, dialysis, ion-exchange chromatography, and paper chromatography. The principles,

method of separation, analysis of the proteins have been studied in this experiment.

16.9 UNIT - END EXERCISES

1. Explain the method of cell lysis.
2. Discuss the importance of protein precipitation and dialysis.
3. Explain the principle and applications of ion-exchange chromatography?
4. How do you determine the MIC and MBC of an antibiotic against the target pathogen?

16.10 SUGGESTED READING

1. Microbiology- A laboratory Manual-International student Edition: 4th edition. By James G. Cappuccino& Natalie Sherman
2. Bacteriological Techniques By F.J. Baker
3. Introduction to Microbial Techniques By Gunasekaran
4. Manual of Industrial Microbiology and Biotechnology, 2nd Edition by Arnold; . Demain and Julian, E. Davie Ronald M. Atlas, Gerald Cohen, Charles L. Hershberger, Wei-Shou Hu, David H. Sherman, Richard C. Wilson and J. H. David Wu.
5. Jennifer M. Andrews. Determination of minimum inhibitory concentrations Journal of Antimicrobial Chemotherapy (2001) 48, Suppl. S1, 5–16

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APPENDIX – 1: MEDIA

1. Nutrient Broth / Agar

Ingredients	Gms / Litre
Peptone	5.000
Sodium chloride	5.000
Beef Extract	1.500
Yeast extract	1.500
Final pH (at 25°C)	7.4±0.2
Agar	15.000

2. Potato Dextrose Agar

Ingredients	Gms / Litre
Potatoes, infusion from	200.000
Dextrose	20.000
Final pH (at 25°C)	5.6±0.2
Agar	15.000

3. Wilkins Chalgren Anaerobic Agar Base

Ingredients	Gms / Litre
Casein enzymic hydrolysate	10.000
Peptic digest of animal tissue	10.000
Yeast extract	5.000
Dextrose	1.000
Sodium chloride	5.000
L-Arginine	1.000
Sodium pyruvate	1.000
Hemin	0.005
Menadione	0.0005
Agar	10.000
Final pH (at 25°C)	7.1±0.2

4. Mueller Hinton Agar

Ingredients	Gms / Litre
Beef infusion from	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.3±0.1

5. Actinomycete Isolation Agar

Ingredients	Gms / Litre
Sodium caseinate	2.000
L-Asparagine	0.100
Sodium propionate	4.000
Dipotassium phosphate	0.500
Magnesium sulphate	0.100
Ferrous sulphate	0.001
Agar	15.000
Final pH (at 25°C)	8.1±0.2

6. Starch Casein Agar

Ingredients	Gms / Litre
Casein	1.00
Starch	10.00
Sea Water	37.00
Agar	15.00
Final pH (at 25°C)	7.2 ±0.2

7. Streptomyces Agar

Ingredients	Gms / Litre
Malt extract	10.000
Yeast extract	4.000
Dextrose	4.000
Calcium carbonate	2.000
Agar	12.000
Final pH (at 25°C)	7.3±0.2

8. Luria Broth/Agar

Ingredients	Gms / Litre
Casein enzymic hydrolysate	10.000
Yeast extract	5.000
Sodium chloride	5.000
Final pH (at 25°C)	7.0±0.2
Agar	15.000

9. Yeast Malt Agar (YM-agar)

Ingredients	Gms / Litre
Peptone	5.000
Yeast extract	3.000
Malt extract	3.000

NOTES

Dextrose	10.000
Agar	20.000
Final pH (at 25°C)	6.2±0.2

10. Minimal Broth/Agar

Ingredients	**Gms / Litre**
Dextrose	1.000
Dipotassium phosphate	7.000
Monopotassium phosphate	2.000
Sodium citrate	0.500
Magnesium sulphate	0.100
Ammonium sulphate	1.000
Agar	15.000
Final pH (at 25°C)	7.0±0.2

11. Starch Agar

Ingredients	**Gms / Litre**
Meat Extract	3.000
Peptic digest of animal tissue	5.000
Starch, soluble	2.000
Agar	15.000
Final pH (at 25°C)	7.2±0.1

12. Citric Acid production media

Ingredients	**Gms / Litre**
Sucrose	140.0
Ammonium nitrate	2.23
Dipotassium ortho phosphate	1.0
Magnesium sulphate	0.23
$CuSO_4$	0.002
$FeCl_3$	0.002
pH	6.5±0.2

13. **YPD Broth (YEPD Broth)**

Ingredients	**Gms / Litre**
Peptic digest of animal tissue	20.000
Yeast extract	10.000
Dextrose	20.000
Final pH (at 25°C)	6.5±0.2

14. **Tryptone Soya Broth**

Ingredients	**Gms / Litre**
Pancreatic digest of casein	17.000
Papaic digest of soyabean meal	3.000
Sodium chloride	5.000
Dextrose	2.500
Dibasic potassium phosphate	2.500
Final pH (at 25°C)	7.3±0.2

Vitamins mix	**(mg/lit)**
Myo-inositol	100.00
Calcium pantothenate	1.00
Thiamin hydrochloride	0.50
Nicotinic acid	2.00
Pyridoxine hydrochloride	2.00
Biotin	0.125
Riboflavin	0.20
Folic acid	0.20

Trace elements	**(µg/lit)**
Manganese (II) chloride tetrahydrate	200.00
Zinc chloride	135 .00
Iron chloride	30.00
Copper chloride	15.00
Boric acid	5.00
Cobalt nitrate hexahydrate	1.00
Sodium molybdate dihydrate	25.00
Potassium iodate	10.00

APPENDIX – II: BUFFERS & REAGENTS

1. Salkowsky reagent

0.5 M $FeCl_3$, distilled water and concentrated H_2SO_4 in a proportion of 1:50:30 (v/v/v). The reagent gives pink colour reaction products; it will disappear within 15 minutes, so complete the experiment (taking absorbance at 530 nm wavelength) within 15 minutes

2. Minimal Salt Solution: (g l^{-1})

KH_2PO_4, 28; $(NH_4)_2SO_4$, 19.6; Urea, 4.2; $MgSO_4$. $7H_2O$, 4.2; $CoCl_2$, 4.2; $FeSO_4$. $7H_20$, 0.07; $MnSO_4$. $7H_2O$, 0.021; $ZnSO_4$ $7H_20$, 0.019; $CaC1_2$, 0.028; if required: yeast extract, 7; and glucose, 15; pH 5.0 ± 0.2.

3. DNS (3,5-dinitrosalicylic acid) reagent

Dissolve 10.6 g of DNS and 19.8 g of NaOH in 1,416 ml of distilled water. After com-plete dissolution, add 360 g of Rochelle salts (sodium potassium tartrate), 7.6 ml of melted phenol (at 50°C), and 8.3 g of sodium metabisulfite, and then mix well. Titrate 3 ml of the DNS reagent using 0.1 M HCl using the phenolphthalein endpoint pH check. It should take 5–6 ml of HCl for a transition from red to colorless. Add NaOH if required (2 g of NaOH added = 1 ml of 0.1 M HCl used for 3 ml of the DNS reagent)

4. Citrate buffer (1 M, pH 4.5)

Dissolve 210 g of citric acid monohydrate in 750 ml of distilled water, and add 50–60 g solid NaOH until pH is 4.3. Dilute the solution to nearly 1,000 ml and check the pH.

5. Citrate buffer (50 mM, pH 4.8)

Dilute 1 M citrate buffer (pH 4.5) by adding 19 times distilled water.

6. Starch solution (1.0%)

Starch solution is prepared freshly by dissolving 1.0 g soluble starch in 100 mL 0.02 M sodium phosphate buffer (pH, 6.9).

7. Potassium Phosphate, buffer 7.5 pH

A 50 mM Potassium Phosphate Buffer, pH 7.5. Prepare using 11.4 mg/mL of potassium phospate dibasic, trihydrate in purified water and adjusting pH with 1 M HCl. This solution is placed at 37°C before use.

8. Casein standard for Protease assay

A 0.65% weight/volume casein solution, prepared by mixing 6.5 mg/mL of the 50 mM potassium phosphate buffer. The solution temperature is gradually increased with gentle stirring to 80-85 °C for about 10 minutes until a homogenous dispersion is achieved. Do not to boil the solution. The pH is then adjusted (7.5) if necessary with NaOH and HCl.

9. Trichloroacetic Acid (110mM)

Diluting a 6.1 N stock 1:55 with purified water. Trichloroacetic acid is a strong acid and should be handled with care.

10. Folin & Ciocalteu's Phenol Reagent [Ready to use]

0.5 M or ½ dilutions.

11. Sodium Carbonate, Anhydrous: A 500 mM Sodium Carbonate solution, prepared using 53 mg/mL of anyhydrous sodium carbonate in purified water

12. Enzyme dilution solution

Sodium Acetate, Trihydrate: An enzyme diluent solution, which consists of 10 mM Sodium Acetate Buffer with 5mM Calcium [Calcium Acetate], pH 7.5, at 37°C. This solution is what we use to dissolve solid protease samples or dilute enzyme solutions.

13. L-Tyrosine, Free Base (1.1mM)

Prepared using 0.2 mg/mL L-tyrosine in purified water and heated gently until the tyrosine dissolves. Allow the L-tyrosine standard to cool to room temperature.

14. 0.05M phasphate, pH 6.9, in 0.12 M NaCl

Dissolve 3.40 g KH_2PO_4 (0.025 moles), 4.35 g K_2HPO_4 (0.025 moles), and 7.0 g NaCl (0.12 moles) in water and dilute to 1.0 1.

MODEL QUESTION PAPER

Lab in Industrial Microbiology & Microbial Biotechnology

Time: 6hrs Max marks:75

Part – I Major experiment. Any one of the following [25 marks]

1. Perform an experiment [A] to screen an extracellular metabolite producing Actinomycetes and write the protocol and principle of quantification of the metabolite.
2. How do you design an experiment [A] to isolate an extracellular enzyme [amylase/protease/lipase] producing bacteria. Write the principle, materials required and the protocol. Interpret your result.
3. How do you perform an experiment [A] to ferment citric acid (i) or wine (ii) in your laboratory? Write the principle and methodology for the assay of citric acid (i) or wine (ii). Interpret your result.

Part – II Minor experiment Any one of the following [15 marks]

1. Perform the experiment [B] to immobilize the microbial cells / Enzymes. Interpret your results.
2. Perform a paper chromatography experiment [B] to separate and identify the aminoacids of the given sample. Value your result.
3. Perform an experiment [B] to calculate the MIC of the given antimicrobial substance (B). Give your comments.

Part – III Identify the Given Specimen [5 x 3 = 15 marks]

C. Solid State Fermentation / Wine Fermentation

D. Any one Enzyme Screening (primary) method/ Antibiosis

E. Dialysis / Ammonium Sulphate Precipitation

F. Ion-exchange chromatography / Gel filtration Chromatography

G. Identification of an industrially important Actinomycetes / Bacteria / Fungi

Part – IV Record Work 10 marks
Part – V Viva 10 marks

Total 75 Marks
